Nutritional ecology of termite-symbionts system using stable isotope techniques(Dissertation_全文)

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Nutritional Ecology of Termite–Symbionts System Using Stable Isotope Techniques

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SUMMARY

In order to answer the question why xylophagous organisms such as termites subsist on a diet containing only a little nitrogen, I studied nitrogen fixation of symbiotic bacteria in the gut of termites using the stable isotope techniques. I measured nitrogen stable isotope ratio ($\delta^{15}N$) of a dry wood termite, *Neotermes koshunensis* Shiraki (Kalotermitidae, Isoptera), together with that of the diet (nesting wood). $\delta^{15}N$ of the termites was lower than that of the diet, showing different pattern compared with other living things. Applying the ^{15}N natural abundance method which has been used in the studies of nitrogen fixation of nodulating plants, it was confirmed that at least 30% to 60% of nitrogen of workers of *N. koshunensis* came from the atmosphere.

Tracer experiment with incorporated $^{15}N_2$ gas confirmed nitrogen fixation of *N. koshunensis* in an experimental condition. The estimated fixation rate of 5.4 years for doubling their nitrogen content was slightly lower than that predicted by the study of natural abundance method, as is often the case of termites in experimental condition generally.

Nitrogen and carbon stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$) of body tissues, mound/nest materials and dietary substrates were determined in termite species with differing trophic habits, sampled from the Mbalmayo Forest Reserve, southern Cameroon (central Africa). $\delta^{15}N$ of termite tissues was gradually enriched along a spectrum of species representing a trophic gradient from wood- to soil-feeding. Species which can be identified from their general biology and from gut content analysis as feeding on well rotten wood or as wood/soil interface feeders showed $\delta^{15}N$ intermediate between sound wood-feeders and soil-feeders. It is proposed that $\delta^{15}N$ is therefore a possible indicator of the functional position of species in the humification process. Differences in $\delta^{13}C$ were also observed between wood-feeding and soil-feeding forms, which suggests the alternative process between acetogenesis and methanogenesis as well as the trophic habit in the humification process. High values of $\delta^{15}N$ in soil-feeding termites suggest that nitrogen fixation was of little importance in these species. A wide range of isotope effects (the difference in isotope ratios between termite and its diet) were observed for both nitrogen ($\Delta\delta^{15}N$= –1.6 to +8.8‰) and carbon ($\Delta\delta^{13}C$= –2.2 to +3.0‰), suggesting a diversity of nutrient acquisition mechanisms within termites and diverse relationships between termites and their intestinal microorganisms.

I analyzed $\delta^{15}N$ and $\delta^{13}C$ of termites sampled from Darwin, northern Australia. High $\delta^{15}N$ values were observed in the *Termes-Capritermes* branch of the subfamily Termitinae and the genus *Amitermes*, two distinct taxonomic groups that evolved from wood-feeding to soil-feeding in Australia. Among eight *Termes-Capritermes* branch species, only two species

(*Xylochomitermes melvillensis* and *Ephelotermes melachoma*) were discernible as wood/soil interface feeders, the remaining six species analyzed were soil-feeders, where the diet preference was referred by $\delta^{15}N$ of workers. The *Termes-Capritermes* group in Australia contained both wood/soil interface feeders and soil-feeders, whereas wood/soil interface feeders in Cameroon were from the *Termes-Capritermes* branch while soil-feeders were from *Cubitermes* group. The result confirmed that soil-feeding forms evolved both in Australia and Africa, but different from phylogenetic groups.

I measured carbon and nitrogen isotope ratios of fungus growing termites sampled in Thailand, Côte d'Ivore and Cameroon. Carbon and nitrogen isotope ratios of fungus growing termites showed the pattern of decomposition of organic matter and the dietary relationships among castes. Preliminary result suggests a possibilily of nitrogen fixation in *Macrotermes subhyalinus.*

Natural abundance method to estimate the contribution of nitrogen fixation was applied to termite species with various feeding habits. It was observed that mound-building grass harvester termites in Australia scarcely utilized atmospheric nitrogen. All soil-feeding species that I studied hardly utilized atmospheric nitrogen as a nitrogen source, whereas nitrogen fixation played an important role in some of wood-feeding species, especially in the dry wood lower termites (the Kalotermitidae).

1. INTRODUCTION

TERMITES are dominant soil animals in tropical terrestrial ecosystems (La Fage and Nutting 1978). The biomass of termites is high, for example approaching 10g m^{-2} in humid forests (Abe and Matsumoto 1979) and they are therefore one of the most abundant animal groups on the earth. In addition, they have a major role in ecosystem processes, especially decomposition. For example, termites consume 24–32% of the annual supply of fallen leaves in Malayan tropical forest (Matsumoto and Abe 1979) and 63% of grass litter in Nigerian savanna, as well as forming a food resource for various predatory animals (Wood and Sands 1978). The basis of the prominence of termites in tropical terrestrial ecosystems lies in their highly developed social organization and symbiosis with microorganisms (Higashi and Abe 1996). Termites are divided into two large groups, lower termites (six families) and higher termites (one family, Termitidae; Wood and Johnson 1986). The lower termites, which harbor associated protozoa in the guts, mainly consume wood, whereas the higher termites, which lack protozoa in the guts and constitute 75% of all species, consume various kinds of dead organic material, including wood, dry grass, bark, lichens, fallen leaves and soil (Wood and Sands 1978).

Abe (1987) distinguished three major life types among termites; one-piece, intermediate and separate type. A piece of wood serves as both a nesting site and food source throughout the lives of one-pieces, whereas it serves separate types as only one of their food items. Intermediate types feed predominantly on the wood within which they nest but do forage elsewhere.

THE LOWER TERMITES consume mainly wood (xylophagous or wood-feeding form), except for the Hodotermitidae, which harvest grass litter in savanna. Xylophagous termites subsist on a diet containing low nitrogen. Most woody tissues contain as little as 0.1% nitrogen and their carbon/nitrogen ratio is typically 300 to 500 (La Fage and Nutting 1978), while xylophagous termites contain about 4 to 10% nitrogen and their C/N ratio is 4-12 (Matsumoto 1976). Whereas, all living organisms require nitrogen for protein and nucleic acid syntheses. The C/N ratio of wood tissue is so high that nitrogen should be a limited resource as compared with carbon. Termites must solve the C/N imbalance problem by either increasing nitrogen-intake or selectively eliminating carbon (Collins 1983, Higashi et al. 1992). Fungus-growing termites (subfamily Macrotermitinae) can concentrate nitrogen and balance C/N ratio by using fungi, which eliminates carbon as CO_2 by respiration (Higashi et al. 1992, Matsumoto 1976). Recycle of nitrogen is another countermeasure to nitrogen

deficiency problem. Ruminants reuse NH_3 as nitrogen source, and nitrogen storage is also important for termites (Potrikus and Breznak 1980a, b, c, 1981). They may recycle stored uric acid though necrophagy and cannibalism (Slaytor and Chappell 1994) and may also digest symbiotic protozoa and/or bacteria.

THE HIGHER TERMITES show remarkable adaptive radiation in feeding habits. Especially, soil-feeding forms, which constitute 51% of all termite genera and 62% of higher termite genera, are found in three out of four subfamilies of the Termitidae (Apicotermitinae, Nasutitermitinae and Termitinae; Noirot 1992) . The soil-feeding habit may therefore have evolved at least three times and constitutes the major trend in higher termite diversification. In addition, a number of higher termites feed on very highly decayed wood in an advanced stage of humification. These are currently known as wood/soil-interface feeders (Eggleton et al. 1995). A second trend is the formation of symbiotic associations with fungi of the genus *Termitomyces* . Higher termites of this type are classified in the subfamily Macrotermitinae (Wood and Johnson 1986). Soil-feeding species are particularly abundant and diverse in forests; for example in Nigerian and Cameroon forests this prominence is largely due to the variety of Apicotermitinae and Termitinae (Wood et al. 1982, Eggleton et al. 1996), whereas in the Brazilian forest, an evolution of soil-feeding appears to have taken place in some Nasutitermitinae (Martius 1994, Eggleton et al. 1994). South Asia region seems to be intermediate between the two regions; soil-feeders are dominated by the Termitinae, but small number of soil-feeders are seen in both Apicotermitinae and Nasutitermitinae (Abe 1987).

In spite of the importance of soil-feeding forms, the feeding habits of soil-feeders and wood/soil interface feeders have hardly been studied because of the technical difficulty in identifying the actual nutrients utilized among complex soil particles. Feeding ecology of soil-feeders has been studied indirectly by the morphology of worker mandible (Sands 1965, Deligne 1966) and gut morphology (Bignell 1994). A recent pilot study of the gut contents by Sleaford et al. (1996) has shown that the diet of soil-feeders is highly heterogeneous, with as many as 10 different item categories, including fine roots, but the most abundant organic material ingested is humus. Wood/soil interface feeders have similar gut contents, but there is more recognizable plant tissue and/or wood fiber in various stages of degradation.

Australian termite fauna is distinct compared with other regions. The *Termes-Capritermes* branch of the subfamily Termitidae (Miller 1994b) is represented in Australia by fifty-four species in thirteen genera (Miller 1991). Information to date suggests they include species whose diets range from wood-feeding to soil-feeding (Hill 1942, Gay 1971, Braithwaite et al. 1988, Miller 1991).

Molecular phylogenetic work has recently been developed to describe the molecular branch preserved in DNA. DeSalle et al. (1992) extracted mitochondrial and nuclear DNA of

Mastotermes electrodominicus preserved in Oligo-Miocene amber. Phylogenetic analysis of fossil and extant 18S rDNA confirmed morphological cladistic analyses of living dictyopterans (Bandi 1995, Kambhampati 1995).

NITROGEN FIXATION, which is catalyzed by nitrogenase, has been demonstrated in termites. Only bacteria including cyanobacteria can fix atmospheric nitrogen. Nitrogen fixation has been observed in many ecosystems; for example, cyanobacteria in the ocean and fresh waters, free living bacteria in the soils, symbionts with legume and non-legume, and symbionts with termites and ruminants. Nitrogen fixation may occur in shipworms (Bivalvia: Teredinidae), in which a cellulolytic nitrogen-fixing bacteria has been shown (Waterbury et al. 1983).

Cleveland (1925) pointed out the possibility that termites can utilize atmospheric nitrogen to explain the fact that they survived on a diet little in nitrogen. Since the sensitive assay of acetylene reduction was established (Breznak et al. 1973, Benemann 1973), many studies using the acetylene reduction assay have shown that termites are able to fix atmospheric nitrogen with the aid of facultative anaerobic bacteria in their hindguts (Breznak 1975, Sylvester-Bradley et al. 1978, Prestwich et al. 1980, Prestwich and Bentley 1981, Breznak 1984, Hewitt et al. 1987, 1990). Similar result has been obtained in *Nasutitermes walkeri* using acetylene reduction (Lovelock et al. 1985) and $^{15}N_2$ incorporation (Chappell and Slaytor 1986), which supported the validity of acetylene reduction assay. Nitrogen fixing bacteria was isolated from termite guts. As expected, nitrogenase activity in termites was associated with the gut where the bacteria were found (Breznak et al. 1973), and the paunch, which was the major site of $^{15}N_2$ incorporation, appeared to be the most likely site of nitrogen fixation (Chappell and Slaytor 1986). French et al. (1976) isolated nitrogen fixing *Citrobacter freundii* strain from Australian termite species (*Coptotermes lacteus, Mastotermes darwiniensis* and *Nasutitermes exitiosus*), and Potrikus and Breznak (1977) isolated another member of the Enterobaceteriaceae (*Enterobacter agglomerans*) from hindguts of *Coptotermes formosanus*.

Experiments with incorporated $^{15}N_2$ gas offered a verification of the assay, confirming the transfer of nitrogen from gut microbes to termite tissues in a caste-selective manner that a greater proportion of the newly fixed nitrogen was translocated to the bodies of workers but to the heads of soldiers (Bentley 1984). The transfer indicates mutualistic relationship between termites and symbionts. The immediate acceptor of ammonia, produced by nitrogenase has not been identified. But, amino acids in high concentration in the paunch were asparagine, glutamate, glutamine, lysine and arginine, and these five amino acids and ammonia constitute 60% of the total amino acids and ammonia in the paunch (Chappell and Slaytor 1986, Slaytor and Chappell 1994). In addition, the $NADP^+$-dependent glutamate

Table 1 Nitrogen fixation (acetylene reduction) rates of live termites (Modified based on Breznak 1984)

Termites	Caste[§]	μg of N fixed (/g fresh wt·day)	TDN (years)[¶]	References
Mastotermitidae				
Mastotermes darwiniensis	W	0.00-23.47	∞-2	French, Turner and Bradbury 1976
Kalotermitidae				
Incisitermes minor	W	1.00-18.87	45-2	Benemann 1973
	S	0.33	137	Benemann 1973
	R	0.66	68	Benemann 1973
Cryptotermes brevis	BL	0.38	119	Breznak, Brill, Mertins and Coppel 1973
Neotermes koshunensis	W	0.08-8.11	357-3	Nakamura and Yara in prep.
	S	0.08-0.37	328-37	Nakamura and Yara in prep.
Termopsidae				
Zootermopsis sp.	W+BL	0.06	753	Breznak, Brill, Mertins and Coppel 1973
Rhinotermitidae				
Coptotermes lacteus	W	0.37-1.87	122-24	French, Turner and Bradbury 1976
Coptotermes formosanus	W	0.16-49.39	238-1	Breznak, Brill, Mertins and Coppel 1973, Breznak 1975
	S	0.03	1507	Breznak, Brill, Mertins and Coppel 1973
	W	3.81-11.27	8-3	Nakamura and Yara in prep.
	S	0.64-1.00	46-29	Nakamura and Yara in prep.
Reticulitermes flavipes	W	0.05	904	Breznak, Brill, Mertins and Coppel 1973
	S	0.02	2260	Breznak, Brill, Mertins and Coppel 1973
Reticulitermes speratus	W	11.52-18.91	3-2	Nakamura and Yara in prep.
	S	1.55-4.71	22-7	Nakamura and Yara in prep.
Rhynchotermes perarmatus	W	3.5	13	Prestwich, Bentley and Carpenter 1980
	S	0.5	90	Prestwich, Bentley and Carpenter 1980
Heterotermes sp.	W	0.94	48	Sylvester-Bradley, Bandeira and Oliveira 1978
	S	0	∞	Sylvester-Bradley, Bandeira and Oliveira 1978
Hodotermitidae				
Hodotermes mosambicus	majorW	0.083		Hewitt, van der Westhuizen, van der Linde and Adam 1987
	minorW	0.08		Hewitt, van der Westhuizen, van der Linde and Adam 1987
Termitidae: Nasutitermitinae				
Nasutitermes corniger	W	6-8	8-6	Prestwich and Bentley 1981, Prestwich, Bentley and Carpenter 1980
	S	0.90-28.40	50-2	Prestwich and Bentley 1981, Prestwich, Bentley and Carpenter 1980

Continue to the next page.

Table 1 *(continued)*

	WC	27.40-81.68	2-0.5	Prestwich and Bentley 1981
Nasutitermes exitiosus	W	0-5.60	∞-8	French, Turner and Bradbury 1976
	W	17.47±4.25		Lovelock, O'Brien and Slaytor 1985
Nasutitermes walkeri	W	34.05±2.1		Lovelock, O'Brien and Slaytor 1985
Nasutitermes takasagoensis	W	1.53-6.30	23-6	Nakamura and Yara in prep.
	S	2.74-11.41	13-3	Nakamura and Yara in prep.
Nasutitermes sp.	W	0.20-13.28	226-3	Sylvester-Bradley, Bandeira and Oliveira 1978
	S	0.87-7.44	52-6	Sylvester-Bradley, Bandeira and Oliveira 1978
	W+S	1.11-18.31	41-2	Sylvester-Bradley, Bandeira and Oliveira 1978
Cornitermes sp.	W+S	1.06	43	Sylvester-Bradley, Bandeira and Oliveira 1978
Labiotermes sp.	W	0.16	282	Sylvester-Bradley, Bandeira and Oliveira 1978
Trinervitermes trinervoides	W	6.86	7	Rohrmann and Rossman 1980
	S	4.48-4.73	10-9	Rohrmann and Rossman 1980
	R	0	∞	Rohrmann and Rossman 1980
Termitidae: Termitinae				
Amitermes sp.	W	0.36	126	Sylvester-Bradley, Bandeira and Oliveira 1978
	S	0.45	100	Sylvester-Bradley, Bandeira and Oliveira 1978
Armitermes sp.	W+S	1.44	31	Sylvester-Bradley, Bandeira and Oliveira 1978
Cubitermes sp.	W	0.17	265	Rohrmann and Rossman 1980
	R	0	∞	Rohrmann and Rossman 1980
Neocapritermes sp.	W	0	∞	Sylvester-Bradley, Bandeira and Oliveira 1978
Procapritermes sp.	W	0.34-0.36	91-85	Nakamura and Yara in prep.
Pericapritermes nitobei	W	0.74	37	Nakamura and Yara in prep.
Termitidae: Macrotermitinae				
Macrotermes ukuzii	W, S, R	0	∞	Rohrmann and Rossman 1980
Odontotermes formosanus	W	0.23-0.65	173-61	Nakamura and Yara in prep.
	S	0.00-0.96	∞-41	Nakamura and Yara in prep.

[§]W, workers(including pseudergates); S, soldiers; BL, brachypterous larvae; R, reproductives; WC, whole colony.

[¶]TDN, time required for termites to double their nitrogen content.

The nitrogen content of all termites is assumed to be 11% (dry wt. basis; Potrikus and Breznak 1980).

dehydrogenase activity in *Coptotermes lacteus* was a bacterial enzyme (Nazarczuk et al. 1981) and catalyzes the incorporation of ammonia into 2-oxoglutarate to produce glutamate. Slaytor and Chappell (1994) stated that this is the typical route for the incorporation of fixed nitrogen into the amino acid pool.

Nitrogen fixation has been demonstrated in all the families of the order Isoptera i.e. the Mastotermitidae, the Kalotermitidae, the Termopsidae, the Hodotermitidae, the Rhinotermitidae and the Termitidae (Collins 1983), but not demonstrated in all subfamilies; e.g. the Macrotermitinae where attempts had been unsuccessful (Rohrmann and Rossman 1980). Recently, Nakamura and Yara (in prep.) have reported that *Odontotermes formosanus* (Macrotermitinae) can fix N_2 a little. Nitrogen fixing activity in various species of termites by acetylene reduction assay is listed in **Table 1** (modified from Breznak 1984). Nitrogen fixing ability is expressed in TDN value, which is defined as the time required for termites to double their nitrogen content. The TDN values showed wide variation of fixation rates in termites intraspecifically as well as interspecifically. Except for some cases, it seems that nitrogen fixation accounts little for nitrogen economy of termites.

One of the reason of this wide variation is due to the difference in nitrogen content of the food. Breznak et al. (1973) found that acetylene reduction rates in *Coptotermes formosanus* varied inversely with the amount of combined nitrogen added to a filter paper. A significant change in fixing rates was detected within five hours of a dietary shift, and variation in fixing rates up to 200-fold were observed. And the difference in fixation rates between *Rhynchomitermes pararmatus* (low) and *Nasutitermes corniger* (high) was attributed to the fact that the former feeds on material richer in combined nitrogen (leaf litter) than does the latter (which feeds on wood litter) (Prestwich et al. 1980). Age or developmental stage of termites may also have a bearing on rates of nitrogen fixation and may account for some of the intraspecific variations. Breznak (1975) found that small worker larvae of *C. formosanus* had 300-fold greater than larger, more fully developed workers. Furthermore, Prestwich and Bentley (1981) obtained the fact that nitrogen fixation by intact colonies of *N. corniger* was higher than that of separated members from the colony **(Table 1)**, which suggests that the metabolism of termites is sensitive to the change of their environment.

Acetylene reduction assay, which is based on the ability of nitrogenase to reduce acetylene to ethylene as well as dinitrogen, is advantageous because it is sensitive, simple assay and real-time measurement is possible (Hardy et al. 1968). However, decline in fixation rate due to the artificial manipulation is dramatic. Lovelock et al. (1985) reported that fixation rate fell to negligible by 24 hours. It is supposed to be inhibited by ammonia (Chappell and Slaytor 1986, Slaytor and Chappell 1994). Moreover, acetylene reduction rate does not always reflect their nitrogen fixation throughout their life by such a short time duration of experiments. Toxicology of acetylene and/or ethylene may also suppress the activity

(Prestwich and Bentley 1982). Accordingly, a different method which is able to reflect the contribution of nitrogen fixation in the natural condition is required. Now, I applied ^{15}N natural abundance method to the study of nitrogen fixation of termites under the natural condition.

STABLE CARBON and NITROGEN ISOTOPE RATIOS have recently been introduced in the study of ecology. Natural abundance isotopic signatures can be used to find patterns and mechanisms at the single organism level as well as to trace food webs, understand palaeodiets and follow whole ecosystem cycling in both terrestrial and marine ecosystems (Lajtha and Michener 1994). The natural abundance of ^{13}C and ^{15}N is expressed in per mil (‰) deviation from standards, which are defined as follows;

$$\delta^{13}C = \left[\frac{(^{13}C / ^{12}C)_{sample}}{(^{13}C / ^{12}C)_{standard}} - 1 \right] \times 1000 \qquad (1)$$

and

$$\delta^{15}N = \left[\frac{(^{15}N / ^{14}N)_{sample}}{(^{15}N / ^{14}N)_{standard}} - 1 \right] \times 1000 \quad , \qquad (2)$$

where PDB (Pee Dee Belemnite) and atmospheric nitrogen are used as the standard, respectively. ^{12}C and ^{13}C content of PDB are 98.889% and 1.111%, and ^{14}N and ^{15}N content of atmospheric nitrogen are 99.634% and 0.366%, respectively (Rundel et al. 1989).

ISOTOPE EFFECT, which is defined as the difference in isotope ratio between animal and its diet, is an essential idea in stable isotope ecology. Carbon and nitrogen isotope effects are defined by the following equations,

$$\Delta\delta^{13}C = \delta^{13}C_{animal} - \delta^{13}C_{diet} \quad , \qquad (3)$$

and

$$\Delta\delta^{15}N = \delta^{15}N_{animal} - \delta^{15}N_{diet} \quad . \qquad (4)$$

In most cases the difference in carbon isotope ratio between animal and its diet is small (<±1‰) and the ratio can therefore be used as a dietary indicator (DeNiro and Epstein 1978). It has been possible to characterize dietary preference of termites between woody and herbaceous material using carbon isotopes (in the case of termites; Boutton et al. 1983, Lepage et al. 1993). The method makes use of the large difference between the stable isotope ratios of C3 plants (woody forms) and C4 plants (grasses) (Fry et al. 1978a, b, Boutton et al. 1978, 1980). Deines (1980) summarized numerous papers and reported the $\delta^{13}C$ values of -

20 to -35‰ for C3 plants and -9 to -14‰ for C4 plants. In detritivorous animals, Martin et al. (1992) pointed out that young *Millsonia anomala* (geophagous tropical earthworm) were able to assimilate young organic matter (fresh organic matter, coarse soil organic matter) as well as fine soil organic matter using $\delta^{13}C$ incorporation (C4 based earthworm to C3 soil).

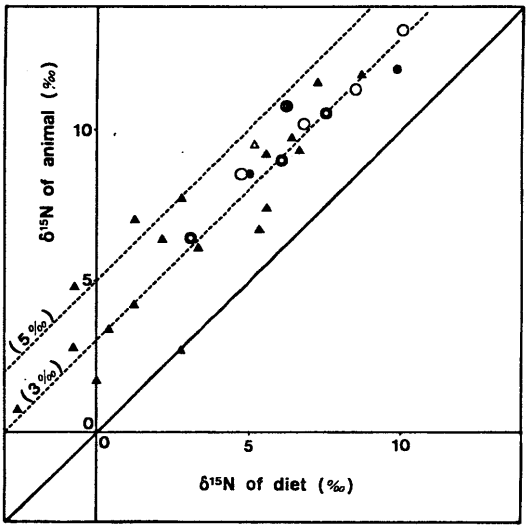


Fig. 1 Relationship between $\delta^{15}N$ of animal and its diet (After Wada et al. 1991).

On the other hand, nitrogen isotope effect is usually positive, averaging 3.4‰ (DeNiro and Epstein 1981, Minagawa and Wada 1984), and since the ratio is assumed to reflect the trophic interaction, nitrogen isotope ratio has been used as a food web indicator for animals (Schoeninger and DeNiro 1984, Ambrose and DeNiro 1986, Wada et al. 1987a, b, 1991, Sealy et al. 1987, Fry 1988, Yoshioka et al. 1994). **Fig. 1** shows the relationship between $\delta^{15}N$ of animal and its diet, summarized by Wada et al. (1991). Another report suggests that 3.4‰ is not always correct for insects, but, $\Delta\delta^{15}N$ is usually non-negative (DeNiro and Epstein 1981). The excretion of low $\delta^{15}N$ as urea or ammonium seems to account for the ^{15}N-enriched nitrogen that is incorporated into animal tissues, presumably due to isotopic processes occurring during amino acid metabolism in the reaction of urea cycle (Steele and Daniel 1978, Minagawa and Wada 1984).

There are several reports that managed to explain isotope effects physiologically. For example, Scrimgeour et al. (1995) observed unexpectedly elevated $\delta^{15}N$ values ($\Delta\delta^{15}N$~10‰) in adult raspberry beetles (*Byturus tomentosus*) collected shortly after emergence from over-wintering sites. $\delta^{15}N$ of feeding larvae was close to that of food plant, red raspberry (*Rubus idaeus*) ($\Delta\delta^{15}N$= 1–2‰), but the elevated $\delta^{15}N$ values in overwintered larvae and adults of raspberry beetle were consistent with an hypothesis of extensive amino acid nitrogen recycling during prolonged fasting (Hobson et al. 1993). Age was shown not to influence on $\delta^{15}N$ of animals such as cattle, pig and marine molluscs (Minagawa and Wada 1984, Sutoh et al. 1987). However, holometabolous insects undergo major developmental changes and attendant changes in feeding behavior with increasing age. Even in hemimetabolous insects, where there is no major change between juvenile and adult forms, food choices may depend on age and development (Behmer and Joern 1993). These factors may affect whole $\delta^{15}N$ values of insects, which should be considered in applying stable

isotope techniques to insects. The other mechanisms may influence isotope effect. A negative correlation was found between $\delta^{15}N$ and anual amount of precipitation for a variety of different species (Heaton et al. 1986, Sealy et al. 1987). Drought tolerant species (mostly browsers) were further found to have $\delta^{15}N$ values 2–4‰ higher than obligate drinkers (mostly grasers) (Ambrose and DeNiro 1986), but the tendency to high $\delta^{15}N$ occurred only in combination with a high C4 diet (Cormie and Schwarcz 1996). They suggested that an increase of $\delta^{15}N$ might result from combined effects from excretion of concentrated urine (to conserve water) and increased internal recycling of nitrogen (to conserve nitrogen), corresponding with the consumption of grasses (Cormie and Schwarcz 1996, Sealy et al. 1987).

BIOLOGICAL NITROGEN FIXATION is another important application of nitrogen isotope techniques. Because of the definition, $\delta^{15}N$ of atmospheric nitrogen is 0. There was virtually no variation in ^{15}N abundance of atmospheric N_2 from broadly dispersed locations in France (Mariotti 1983). Whereas, $\delta^{15}N$ of soil nitrogen varies from site to site depending upon the nitrogen sources and dynamics of nitrogen including denitrification. $\delta^{15}N$ of plants is influenced by soil nitrogen and other factors (Handley and Raven 1992).

$\delta^{15}N$ of a living organism, when it takes up two sources of nitrogen that have different $\delta^{15}N$, reflects the fractional contribution of the two sources ("Two sources and common sink" model; Shearer and Kohl 1993). Actually, contribution of nitrogen fixation to legume plants has well been studied (Amarger et al. 1979, Kohl et al. 1979, 1980, Mariotti et al. 1983, Shearer and Kohl 1986). In the case of legume plants, the fraction of nitrogen derived from atmosphere ($\%N_{dfa}$) is calculated by the following equation,

$$\%N_{dfa} = \frac{\delta^{15}N_x - \delta^{15}N_{Sample}}{\delta^{15}N_x - \delta^{15}N_f} \times 100 \quad , \qquad (5)$$

where $\delta^{15}N_x$ is the isotope ratio if all of nitrogen comes from soil, $\delta^{15}N_{sample}$ is the isotope ratio of sample and $\delta^{15}N_f$ is the isotope ratio if all of the nitrogen comes from atmosphere. In the case of nodulating plants, $\delta^{15}N_x$ is given by non-nodulating plants (reference plants) and $\delta^{15}N_f$ is given by the nodulating plants grown in nitrogen-free medium.

In the study of legume plants, reference plants must be selected carefully whose phenology and nutrient absorbing site correspond to fixing plants. Precision of estimation of nitrogen derived from atmosphere depends on $\delta^{15}N$ value of reference plant that reflects nitrogen source in the soil. Since fractionation during biological nitrogen fixation exhibits rather narrow range from -2.0‰ to -0.2‰ for nodulating plants (Yoneyama 1987) and other nitrogen fixing bacteria (Wada et al. 1986, Shearer and Kohl 1993), the higher $\delta^{15}N$ value of reference plant is, the more precise estimation becomes.

^{15}N NATURAL ABUNDANCE IN FOREST ECOSYSTEMS vary in both organic N and inorganic N associated with pedogenic process (Nadelhoffer and Fry 1994). Nitrogen in plant tissue typically has $\delta^{15}N$ values ranging from about -5 to 2‰ (Fry, 1991), but is dependent on local nitrogen cycling. For example, –10‰ of $\delta^{15}N$ was reported for foliage on very young soils in Hawaiian forests (Vitousek et al. 1989) and +10‰ in foliage of desert woodland (Virginia et al. 1989). Nitrogen input to the soil is mainly by nitrate and ammonia deposition and by biological nitrogen fixation. $\delta^{15}N$ of ammonia and nitrate are depleted (typically -10 to 0‰) on average, and those of nitrogen by N_2-fixation are slightly negative (Yoneyama 1996).

In general, tree tissues and fresh litter are slightly depleted in ^{15}N relative to soils and $\delta^{15}N$ values increase with depth in soil profiles to about +8 ± 2‰ at depth of 20–40 cm (Delwiche and Steyn 1970, Riga et al. 1971, Rennie et al. 1976, Shearer et al. 1978, Létolle 1980, Mariotti et al. 1980b, Wada et al. 1984, Ledgard et al. 1984, Shearer and Kohl 1986, Nadelhoffer and Fry 1994). Ammonia volatilization (Högberg 1990) and denitrification (Mariotti et al. 1981, 1982, 1988) accounts for the evolution of $\delta^{15}N$ in the decomposition process. Experiments with incorporated plant litter confirmed the increase of $\delta^{15}N$ value of soils, caused by the partial loss of ^{15}N-depleted volatile N compounds (amine and ammonia, Turner et al. 1983) and by leaching of ^{15}N-depleted compounds, including by denitrification (Nadelhoffer and Fry 1988).

Tiessen et al. (1984) studied natural nitrogen isotope ratio of soil organic matter associated with organo-mineral particle size fractions of two cultivated and two native grassland soil. They reported that fine clay (<0.2μm) had about 7‰ higher $\delta^{15}N$ than total nitrogen. The technique was based on the soil fractionation by ultrasonification and centrifugation/decantation (Jackson 1956, Anderson et al. 1981, Tiessen and Stewart 1983, Ledgard et al. 1984, Balesdent et al. 1991). Mineralization of relatively ^{15}N-depleted nitrogen and reassimilation of relatively ^{15}N-enriched nitrogen during microbial N turnover may result in ^{15}N enrichment in acid hydrolyzable fractions, representing labile N (proteinous) materials, compared to non-hydrolyzable fractions resistant to microbial cycles (Cheng et al. 1964, Selles et al. 1984). Stable isotope ratios at microsites in soil organic matter are still difficult to measure, and it is required to develop techniques to cope with. However, $\delta^{15}N$ showed no spatial variety among non-fixing trees in a forest (Shearer et al. 1978, Sutherland et al. 1991), which suggests the availability of measuring the isotope ratios in a field site without referring to each habitat of a colony of termites.

IN THIS STUDY, I applied stable isotope techniques to the study of termite-symbionts system. First, I applied the ^{15}N natural abundance method to evaluate the nitrogen fixation in

the nitrogen economy of *Neotermes koshunensis* (Kalotermitidae, Isoptera), which nests in a dead branch and consumes the same wood. I conducted $^{15}N_2$ tracer experiment with *N. koshunensis* to compare with ^{15}N natural abundance method.

Secondly, I measured natural nitrogen and carbon isotope ratios of termites collected in Cameroonian seasonal forest and established a methodology to study humivorous termites. And then, I applied this technique to Australian termites. I combined stable isotope techniques and molecular phylogenetics to study the evolution of soil-feeding forms in Australia, and compared the phylogenetic pattern with Cameroon. Fungus growing termites, Australian wood-feeders and grass-harvesters were studied by stable isotope techniques and preliminary discussion was made.

Finally I applied the natural abundance method of estimating nitrogen fixation to various taxonomic and nutritional groups of termites, and discussed the importance in the nitrogen economy. I discussed a possibility of the use of stable isotope techniques in the study of detritivorous animals.

2. Stable Isotope Techniques

Samples were converted to CO_2 and N_2 gas through the dry combustion method (Minagawa et al. 1984, Watanabe and Wada 1993) and then carbon and nitrogen isotope ratios were determined by a mass spectrometer.

Sample preparation

Samples of termites, grass, wood tissue, soil and mound material were well dried by vacuum dryer at 60°C or freeze dryer for at least 24 hours, and were kept dried until the procedure mentioned below. Wood and soil samples were ground into a fine powder, using a bowl mill. Carbonates were not present in the soil samples and subsequent acidification therefore did not affect the carbon isotope composition. Subsamples were then weighed (in the case of termites, several individuals) and sealed in quartz tube as follows.

Combustion and purification

1. The combustion apparatus consist of a double quartz tube (9mm diameter(ϕ) 300mm length(l) and 6mm(ϕ) 75mm(l)), which is preheated at 850°C for 2 hours to eliminate organic contaminants. Sample of termites (typically 3–10 mg) is placed in the inner tube **(Fig. 2, left)**. One gram of CuO wire pieces (purified for 2 hr at 850°C) is placed into the tube followed by a piece of Ag foil (0.05mm thick, 4 × 25 mm, preheated 850°C). The tube is then put into the outer tube with 0.5g of Cu wire.

1'. Grass or wood tissue (typically 100–150 mg) is placed directly in the combined quartz tube (9mm(ϕ) 150mm(l) combined to 25mm(ϕ) 150mm(l)),together with 8g of CuO wire (Also they were preheated at 850°C for 2 hours) followed by a inner tube including 1g of Cu and Ag foil

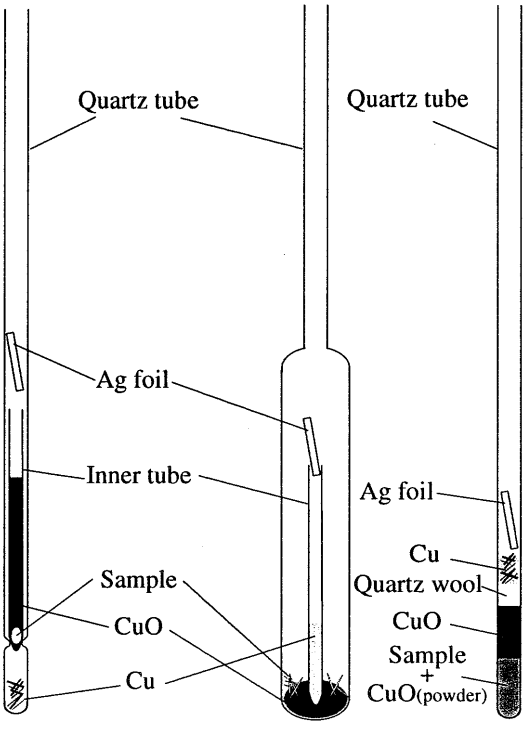


Fig. 2 Combustion apparatus made of quartz tube
left: for termites center: for wood and grass
right: for soil and mound material

(Fig. 2, center).

1''.Soil or mound material sample (typically 100–200 mg), which has been well mixed together with 1g of CuO powder, is placed directly in the combusted quartz tube (9mm diameter(ϕ) 300mm length(l), **Fig. 2, right**). One gram of CuO wire pieces and quartz wool (Also they were preheated at 850°C for 2 hours) are placed into the tube followed by 1g of Cu and a piece of Ag foil.

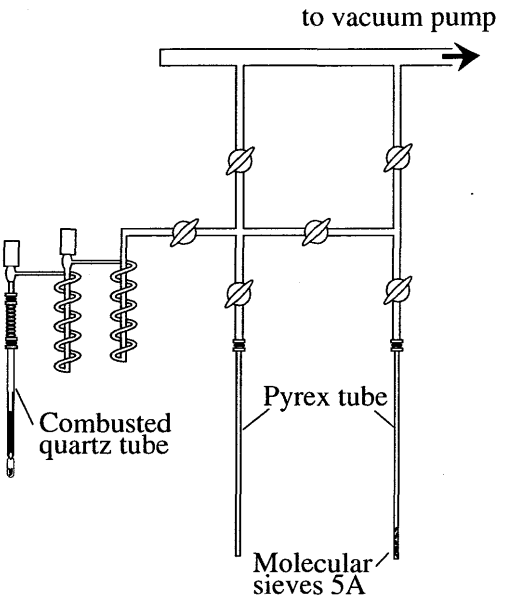


Fig. 3 Vacuum system for purifying N_2 and CO_2 of combusted quartz tube

2. The whole tube is attached to a vacuum manifold via a Cajon fitting and evacuated several hours to remove air and water in a sample and then sealed in 25cm length.
3. The sample tubes of termites are then combusted in a muffle furnace to 500°C for $^1/_2$ hr and then elevated to 850°C for 2hr. And the tubes of grass, wood tissue, soil and mound material are combusted at 500°C for 1 hr and then elevated to 850°C for 4hr. The sample tube in the furnace is allowed to cool for more than 10hr so as to convert CO to CO_2 and NO_x to N_2 at low temperatures. After combustion, the tube is cooled at -20°C until the purification.
4. The tube is attached to a vacuum manifold and cracked to introduce the N_2 gas using a metal fitting as illustrated in **Fig. 3**. CO_2 and H_2O in the sample tube are condensed by dipping the bottom of the tube into liquid nitrogen.
5. N_2 gas is concentrated in a vacuum system by using a mercury Toepler pump or a molecular sieves trap, and sealed in a 6mmϕ Pyrex tube (preheated for 2hr at 450°C) for $^{15}N/^{14}N$ analysis.
6. CO_2 gas is concentrated in a 6mmϕ Pyrex tube (preheated for 2hr at 450°C) which was dipped into the liquid nitrogen trap in a vacuum system, and sealed for $^{13}C/^{12}C$ analysis.

Mass spectrometry

The samples of nitrogen and carbon were measured by a double collector mass spectrometer (Thermoquest Delta S and MAT252). Accuracy of the entire procedure was within ±0.10‰ for carbon and ±0.15‰ for nitrogen, respectively.

3. NITROGEN FIXATION IN *NEOTERMES KOSHUNENSIS*

Neotermes koshunensis Shiraki (Kalotermitidae, Isoptera) is a dry-wood lower termite, distributing in Ryukyu Islands, Formosa and China. Their nests are made mainly in the dead branches of living trees (Ikehara 1966), and workers (pseudergates) consume the nesting wood for diet. Whereby, their food source is the nesting wood and easily identified.

In the first section, I presented the stable isotope natural abundance method to estimate the contribution of nitrogen fixation in *N. koshunensis*. Then, I compared the results with tracer experiment.

3.1 Natural abundance method

3.1.1 Sampling sites

Samples were collected in August 1992 at Mihara region of Iriomote Island in southern part of Japan (**Fig. 4**). Nesting plant of Colony 1 was about 7cm in diameter and 70cm in length, and those of Colony 2–6 were 3–5cm in diameter and 30–50cm in length (**Table 2**). Nesting plant of colony 1 was unidentified and those of 2–6 were *Bruguiera gymnorrhiza*, a mangrove tree, in a swamp forest.

Termites, wood tissue and feces (for Colony 2–6) were collected and dried at 60C°. Larvae of longicorn beetle (species unidentified: Cerambycidae, Coleoptera) found in the wood containing Colony 1 were also collected for comparison.

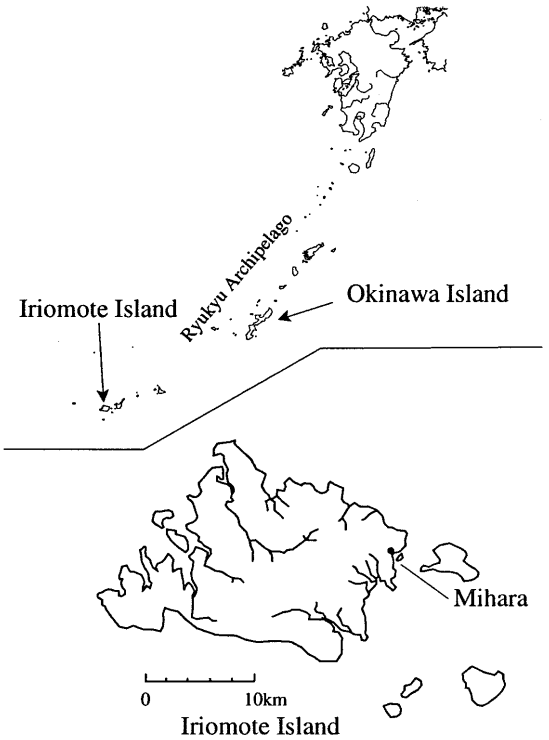


Fig. 4 Study sites in Ryukyu Archipelago : Okinawa Island (26°30' N, 128° E) and Iriomote Island (24°20' N, 123°50' E)

3.1.2 Results

Results of the analysis are shown in **Table 2**. $\delta^{15}N$ of wood tissue were from 0.5‰ (colony 3) to 2.9‰ (colony 2), whereas $\delta^{15}N$ of workers were from -0.5‰ (colony 1)

Table 2 $\delta^{15}N$ values of six colonies of a dry wood termite, *Neotermes koshunensis*. Samples were collected at Iriomote Island in Japan in August 1992. Nesting plant of colony 1 was unidentified, those of 2-6 were *Bruguiera gymnorrhiza* in the mangrove forest. $\delta^{15}N$ of wood tissues is shown in the averaged values (mean±s.e.(‰)) of triplicates. $\delta^{15}N$ of workers (pseudergates) and soldiers are shown in the averaged values (mean±s.e.(‰)), where measurement numbers are shown in the parentheses. In each measurement, several workers or soldiers were put together.

Colony number	$\delta^{15}N$ of wood tissue (‰)	$\delta^{15}N$ of workers (‰)	$\delta^{15}N$ of soldiers (‰)	$\delta^{15}N$ of feces (‰)
1	1.3±0.3	-0.5±0.1 (3)	-1.6±0.1 (2)	-
2	2.9±0.3	0.3±0.1 (2)	not measured	1.8
3	0.5±0.2	-0.2±0.1 (4)	-2.4 (1)	-0.2
4	2.0±0.3	0.6±0.1 (5)	-0.5 (1)	0.6
5	2.2±0.1	0.8±0.2 (4)	-0.5 (1)	1.5
6	2.4±0.1	-0.4±0.2 (7)	-2.4±0.1 (2)	-0.5

to 0.8‰ (colony 5) and $\delta^{15}N$ of soldiers were from -2.4‰ (colony 6) to -0.5‰ (colony 4, 5). $\delta^{15}N$ of feces collected from the nest were from -0.5‰ (colony 6) to 1.8‰ (colony 2). The relationship between $\delta^{15}N$ of wood tissue and termites is shown in **Fig. 5**. Each plot indicates the six colonies I studied. $\delta^{15}N$ of workers were always lower than that of wood tissue by 0.7–2.8‰ and $\delta^{15}N$ of soldiers were always lower than that of workers by 1.1–2.2‰.

3.1.3 Estimation of the fractional contribution of nitrogen fixation in *N. koshunensis*

Applying equation (5) to the case of workers of termites, the fraction of nitrogen derived from atmosphere ($\%N_{dfa}$) is given by the equation (6),

$$\%N_{dfa} = \frac{\left(\delta^{15}N_{wood} + \Delta_{dig}\right) - \delta^{15}N_{sample}}{\left(\delta^{15}N_{wood} + \Delta_{dig}\right) - \Delta_{fix}} \times 100 \quad , \qquad (6)$$

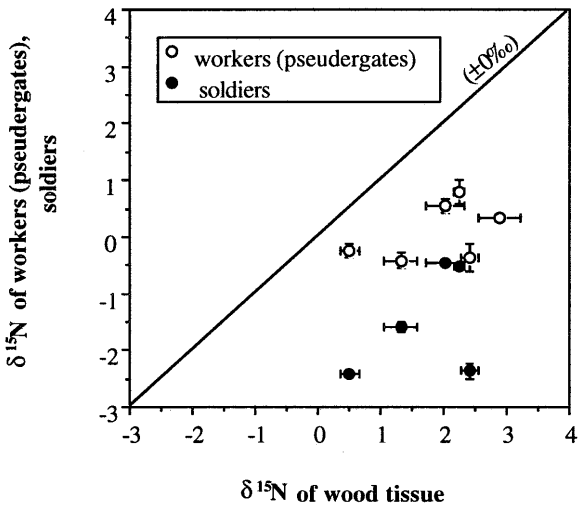


Fig. 5 Relationships between $\delta^{15}N$ of diet (‰) and $\delta^{15}N$ of workers and soldiers (‰) of *N. koshunensis*. Open circle shows $\delta^{15}N$ of workers (pseudergates) and closed circle shows that of soldiers, with standatd error of several measurements. When no isotopic shift occurs during digestion, points would be on the slope 1 shown as (±0‰).

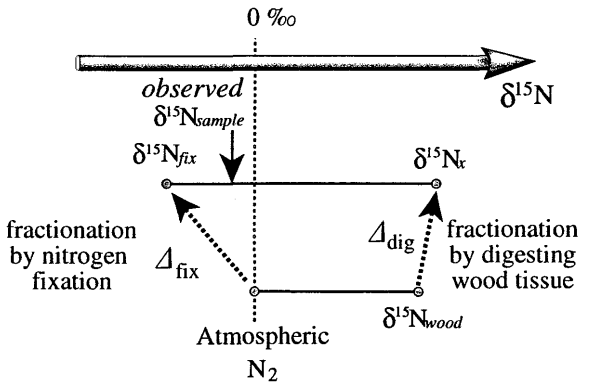


Fig. 6 Schematic diagram of two source model for estimating the fractional contribution of nitrogen fixation in termites. Atmospheric nitrogen ($\delta^{15}N=0$) and wood ($\delta^{15}N_{wood}$) are the sources of nitrogen in termites. $\delta^{15}N$ of atmosphere is lowered to $\delta^{15}N_{fix}$ by fractionation of nitrogen fixation while $\delta^{15}N_{wood}$ is highered to $\delta^{15}N_x$ by fractionation of digestion. $\delta^{15}N_{sample}$ is at a point between $\delta^{15}N_{fix}$ and $\delta^{15}N_x$, depending on the ratio of two sources utilized.

where, Δ_{dig} is the fractionation during digesting wood tissue, Δ_{fix} is the fractionation occurring as a result of nitrogen fixation and $\delta^{15}N_{sample}$ is isotope ratio of the sample.

Illustrative explanation of the equation (6) is shown in **Fig. 6**. $\delta^{15}N_x$ should be obtained if all of nitrogen comes from the wood and $\delta^{15}N_{fix}$ should be obtained if all of the nitrogen comes from the atmosphere. $\delta^{15}N_{sample}$ lies on a point between $\delta^{15}N_x$ and $\delta^{15}N_{fix}$, depending on the ratio of the two sources utilized.

Table 3 The isotope fractionation associated with N_2 fixation*

Host	Type§	Number of species	Mean observed fractionation (‰)
Azotobacter	b	4	-1.2
Glycine max	l		-1.5
Medicago sativa	l		0.2
Trifolium	l	2	0.5
Vicia faba	l		0.2
Lupinus	l	2	0.0
Phaseolus vulgaris	l		-1.5
Cyamopsis tetragonolaba	l		0.8
Dalea	l	2	-1.7
Prosopis glandulosa	l		-1.5
Lotus pendunculatus	l		-0.1
Macroptillium atropurpureum	l		-3.4

*Summary of data was calculated by Shearer and Kohl (1993) using the references published by Bergersen and Turner (1983), Delwiche and Steyn (1970), Domenach and Corman (1984), Hoering and Ford (1960), Kohl and Shearer (1980), Mariotti et al. (1980a), Shearer et al. (1983), Shearer and Kohl (1986), Steele et al. (1983).
§ b: nitrogen fixing bacteria, l: legume plant.

Table 4 The fraction of nitrogen derived from the atmosphere (%Ndfa) calculated from the data of workers (Table 2) with the ranges of Δ_{dig} and Δ_{fix} in the text; Δ_{dig} >0‰, -2‰< Δ_{fix} <0‰.

Colony number	%Ndfa (%)
1	>50
2	>50
3	>30
4	>40
5	>30
6	>60

Since the values of the isotope fractionation: Δ_{dig} and Δ_{fix} for this termite have not been measured, these values were estimated as follows.

As mentioned in Chapter 1 (**Fig. 1**), Δ_{dig} is generally positive, averaging 3.4‰ (DeNiro and Epstein 1981, Minagawa and Wada 1984). In my analysis, the $\Delta\delta^{15}N$ of larvae of longicorn beetle was 0.8‰ on the average, which showed the same tendency. As there was no further information, I assumed Δ_{dig} positive.

Whereas, the $\delta^{15}N$ value of termites was lower than that of their food. If feces had higher $\delta^{15}N$, it could be explained only by excreting nitrogen of high isotope ratio. However, the $\delta^{15}N$ of termite feces collected in the nests were lower than that of their food by 0.7–2.9‰ (**Table 2**). Whereby, there is no explanation for this phenomena except for dilution effect by atmospheric nitrogen.

Fractionation during biological nitrogen fixation exhibits rather narrow range from –2.0‰ to –0.2‰ for legume plants (Yoneyama 1987, Ledgard 1989), -1.9 and -1.8‰ for *Frankia* infected *Alnus* (Domenach et al. 1989). *Azolla-Anabaena* symbioses had a fixed N of -1.4‰ (Yoneyama et al. 1987) and free-living N-fixing bacteria *Azotobacter* had -1.2‰ on the average (**Table 3**). In total, Δ_{fix} shows slightly negative values irrespective of species. Therefore, I assumed $-2‰ < \Delta_{fix} < 0‰$.

Substituting these ranges of Δ_{dig} and Δ_{fix} into the equation (3), the fraction of nitrogen of workers derived from atmosphere ($\%N_{dfa}$) was calculated (**Table 4**) . At least 30% to 60% of nitrogen of the termites was derived from atmospheric nitrogen.

3.1.4 Discussion

Since $\delta^{15}N$ value itself has no information of time, it is not easy to exchange $\%N_{dfa}$ to TDN values directly. However, TDN of 0.5-2, which was the highest value obtained for a whole colony of *Nasutitermes corniger* (**Table 1**), would account for 67-17% of the annual nitrogen requirement if the production/biomass ratio of 3:1 for the termites of the subfamily Nasutitermitinae is assumed (Collins 1983). That is, the lowest TDN values down to 0.5 or 1 must be consistent with my estimation of $\%N_{dfa}$, although the estimated values of TDN of

other species varied widely.

The wide ranges of TDN by acetylene reduction assay (**Table 1**) may be due to the artificial manipulation and the short time duration of experiments in the acetylene reduction assay. And integrated contribution of nitrogen fixation is not always consistent with the activity, because there should be variation in the requirement of nitrogen among stages (Breznak 1984).

3.2 $^{15}N_2$ tracer method

In the last section, the rate of nitrogen fixation could not be obtained. And so, in order to estimate the rate of nitrogen fixation of *N. koshunensis* in an experimental condition, I kept termites in vial bottles. This study is to be available to an estimation of fractionation factors for this species.

3.2.1 Methods

The sample termites of *N. koshunensis* were collected in Okinawa Island (**Fig. 4**) on May 20, 1993 by Mr. K. Sugio. They nested in *Ligustrum japonicum* .

Tracer experiment

Seven workers (pseudergates) were kept in a 69 ml vial bottle together with nesting wood (series A, B and D) or filter paper (Advantec No. 6, Toyo Roshi Co. Ltd.) (series C) for food (**Table 5**). Inner tube (9mm diameter) filled with 1 ml of saturated NaOH*aq* was fixed at the bottom of the bottle to absorb respired CO_2. The bottle was sealed with butyl rubber stopper and fixed on a 300ml beaker filled with water (**Fig. 7**), which prevent N_2 from intruding into the bottle. 2 ml of $^{15}N_2$ tracer (A Matheson®, 99.7% in ^{15}N atom %) was injected in the bottles of series A and C. As a control to the change in pressure, series D, with nesting wood and being kept unsealed in a vial bottle, was also incubated (**Table 5**).

The samples were incubated at 25°C until the analyses of the stable isotope ratios at 4, 8, 12 and 20 weeks after June 16, 1993. On each time, gas samples and termites of the two bottles of each series

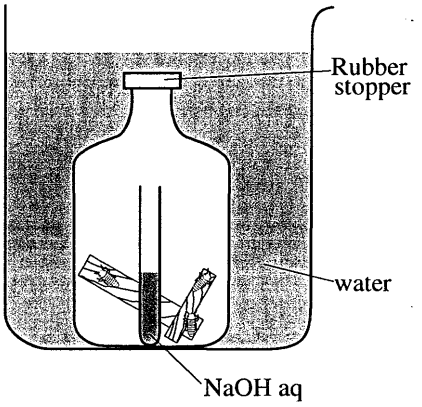


Fig. 7 Experiments with $^{15}N_2$ tracer and Ar/O_2 gas. Termites were placed in a vial bottle (69ml) together with wood tissue or filter paper and NaOH*aq*, and then the bottle was sealed with rubber stopper. Bottle was dipped into water except for series D (see Table 5).

Table 5 Procedure of tracer study and Ar/O_2 experiment

series	food	air	incubation period
A	nesting wood*	^{15}N enriched air#	4, 8, 12, 20 weeks
B	nesting wood*	natural air	4, 8, 12, 20 weeks
C	filter paper	^{15}N enriched air#	4, 8, 12, 20 weeks
D	nesting wood*	open air	4, 8, 12, 20 weeks
E	nesting wood*	Ar/O_2	8, 14 weeks
F	nesting wood*	natural air	8, 14 weeks

*$\delta^{15}N$ of nesting wood tissue was -3.5±0.3‰ (s.e.).
^{15}N enriched air contained 4% ^{15}N (atom%).

were sampled for the analyses.

During the incubation period, 50μl of each bottles of series B was sampled to analyze oxygen concentration by TCD-gas chromatography once a week, and oxygen was injected into the incubated bottle to adjust the concentration to 21%. Water (0.1ml) was also injected to keep humidity.

Ar/O_2-gas experiment

Seven termites of *N. koshunensis* were fed on the nesting wood in Ar/O_2-gas (Ar 79%, O_2 21% v/v) (series E) and in the air (control, series F) (**Table 5**). Getting started on September 11 1993, the samples were incubated at 25°C during the period of 8 or 14 weeks. Termites in the two bottles of each series were sampled for the analyses at 8 and 14 weeks. During the incubation period, the bottles were flushed with Ar/O_2-gas (series E) or open air (series F) and water (0.1ml) was injected once a week.

Mass spectrometry

Gas samples were sealed in a 6mmϕ Pyrex tube (preheated for 2hr at 450°C) with 2.0g of Cu and combusted at 400°C for 4hr to remove oxygen. After purification, N_2 gas was concentrated in a vacuum system by using a molecular sieves trap (**Fig. 2**). N_2 gas was sealed in a 6mmϕ Pyrex tube for ^{15}N analysis. The samples were measured by single scanning method of a mass spectrometer (Thermoquest Delta S).

Contribution of nitrogen fixation in the experimental condition

In the series A, nitrogen fixed during the experiment was calculated as follows. Nitrogen in termites at time t is regarded as the mixture of nitrogen in the initial state together with that derived from wood tissue ($1-f$), and nitrogen derived from labeled air (f). ^{15}N budget in this two source model is given as: $(1-f)R'_{control} + fR'_{atmosphere} = R'_{sample}$, where $R' = {}^{15}N/({}^{14}N + {}^{15}N)$. In the series C, f is the net nitrogen acquisition of nitrogen and is calculated as the same. Then fraction of nitrogen that was fixed during the experimental

period t is give by (Warembourg 1993),

$$f(t) = \frac{R'_{\text{sample}} - R'_{\text{control}}}{R'_{\text{atmosphere}} - R'_{\text{control}}} \quad , \qquad (7)$$

where sample represents series A or C, and control represents series B. As the total amount of N in the sample was measured, it was possible to calculate the real amount of N fixed during the exposure period.

Fractionation factor during digesting wood tissue (Δ_{dig}) is given in Ar/O_2 experiment. Without utilizing atmospheric nitrogen, $\delta^{15}N$ of termites will converge on the value of $\delta^{15}N_{wood}+\Delta_{dig}$.

3.2.2 Results

$\delta^{15}N$ of wood tissue used in the experiment was -3.5±0.3‰. During the incubation

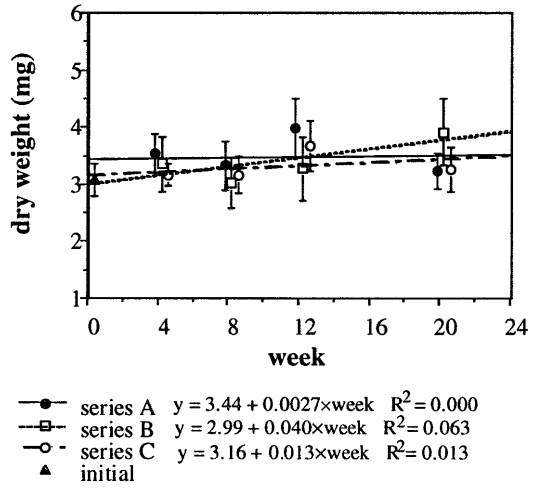


Fig. 8 Changes in body weight of termites during the experiment

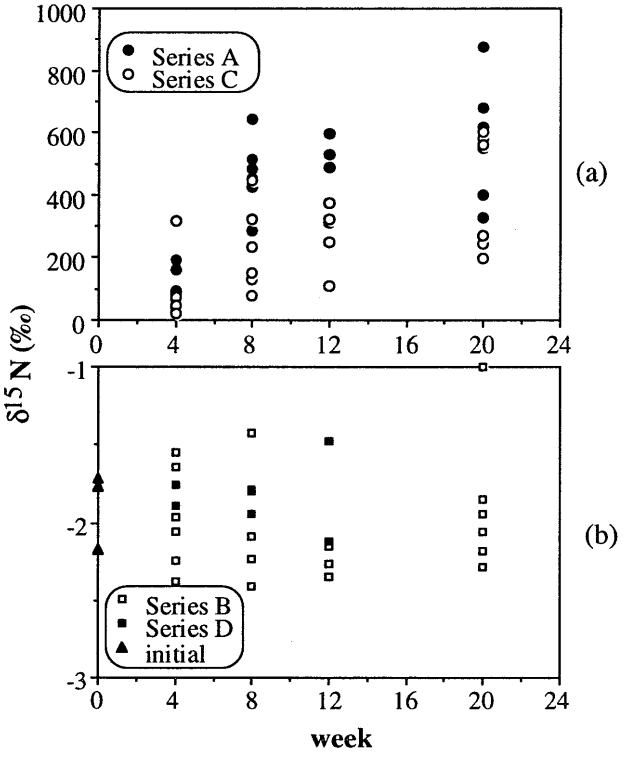


Fig. 9 Changes in $\delta^{15}N$ values during the experimental period for *tracer study*. Termites with enriched ^{15}N air (series A and C) are shown in figure (a), and termites for control (series B and D) are shown in figure (b) together with initial values (closed triangle).

period, dry weight of termites scarcely changed (**Fig. 8**). Variation among individuals was very large.

Rate of nitrogen fixation under an experimental condition

Every sampling time, two bottles of each series were unsealed, and stable isotope ratios of both atmosphere and three individuals in each bottle were analyzed. As shown in **Fig. 9**, $\delta^{15}N$ of series A and C increased during the incubation period. In most cases, $\delta^{15}N$ of series A was higher than that of series C.

Then the fraction of nitrogen derived from labeled air [$f(t)$] were calculated for series A and C, using the equation (7) (**Fig. 10**). The rate of N_2 fixation is $N_c \cdot \left[\frac{df(t)}{dt}\right]_{t=0}$ (mgN/mg termite·*time*), where N_c is the nitrogen content of termite. To obtain TDN values, the value of $\left[\frac{df(t)}{dt}\right]_{t=0}$ was calculated from the slope of the linear regression line. The regression line of $f(t)$ was calculated as a proportional function of time (week), using the data of 4, 8, 12 and 20 weeks. Slope was 0.0036 $week^{-1}$ (r=0.64) for series A and 0.0024 $week^{-1}$ (r=0.64) for series C (**Fig. 10**). TDN is defined as time for doubling their nitrogen content, which is calculated as $1/\left[\frac{df(t)}{dt}\right]_{t=0}$. Therefore, I converted to the TDN values during the exposure period, using TDN (year)=1 / slope ($week^{-1}$) / 52 (weeks). I wrote down the averaged TDN value that was calculated from the regression line mentioned above, together with the values calculated from each points using TDN (year)=1 / $f(t)$ / (52 / exposure period(weeks)) in **Table 6**.

The averaged fixing rate given in TDN values were 5.4 years for series A and 8.1 years for series C. Termites fed on the nesting wood (series A) ranged 3.8–11.1 years (calculated from 4 weeks data), 2.3–5.0 years (8 weeks) 3.8–5.8 years (12 weeks) and 4.4–11.6 years (20 weeks). And, termites fed on paper filter (series C) ranged 2.3–33.0 years (4

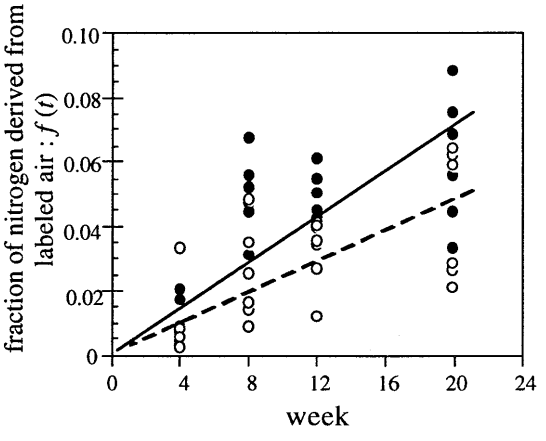


Fig. 10 Changes in fraction of fixed nitrogen [$f(t)$] and regression lines

$$f(t) = \frac{R'_{sample} - R'_{control}}{R'_{atmosphere} - R'_{control}}$$

where $R' = {}^{15}N/({}^{15}N + {}^{14}N)$

Table 6 TDN values during the exposure period
Average TDN values were calculated by using the slope of regression line in Fig. 10*. TDN values of each duration were calculated by the equation mentioned below#.

Series (Food)	TDN (years) average*	4 weeks#	8 weeks#	12 weeks#	20 weeks#
Series A (Nesting wood)	5.4	3.8– 11.1	2.3– 5.0	3.8– 5.8	4.4– 11.6
Series C (Filter paper)	8.1	2.3– 33.0	3.2– 17.6	5.6– 19.9	6.0– 18.2

* TDN (year)=1 / slope of regression line in Fig. 10 / 52 (weeks).
# TDN (year)=1 / *f(t)* / (52 / exposure period(weeks)), where *f(t)* is the fraction of nitrogen derived from labeled air.

weeks), 3.2–17.6 years (8 weeks), 5.6–19.9 years (12 weeks) and 6.0–18.2 years (20 weeks).

Termites for control (series B) and for pressure control (series D) did not change during the experimental period (**Fig. 9**).

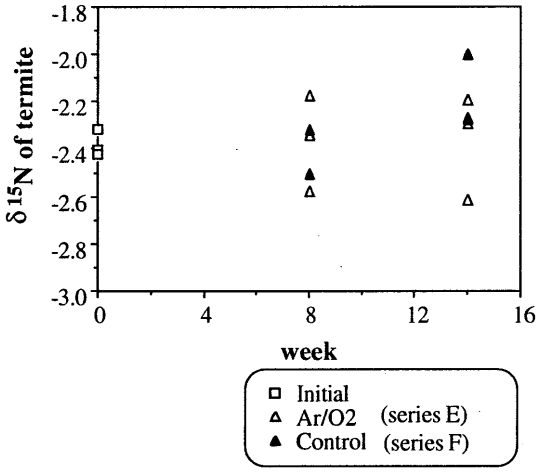


Fig. 11 Changes in $\delta^{15}N$ values during the Ar/O_2 experiment

Ar/O_2-gas experiment

Every sampling time, two bottles of each series were unsealed, and stable isotope ratio of two individuals in each bottle was analyzed (**Fig. 11**). There were no difference between Ar/O_2-gas (series E) and control (series F) in both sampling time of 8 and 14 week.

3.2.3 Discussion

Rate of nitrogen fixation under the experimental condition

The TDN values of *N. koshunensis* has been reported to be 3-357 years (0.08–8.11 μg of fixed N / g fresh wt·day) for workers by using the acetylene reduction assay (Nakamura and Yara in perp; **Table 1**). My experimental data showed rather narrow range than their estimation of 2.3–11.6 years (4.1–21 μg of fixed N / g fresh wt·day, where I assumed nitrogen content of *N. koshunensis* was 7%) (**Table 6**). This difference must be due to the artificial manipulation of acetylene reduction caused by 1) shorter time duration of experiment rather than my experiment, 2) decline in fixation rate reported by Lovelock et al. (1985), and 3)

toxicology of acetylene and/or ethylene (Prestwich and Bentley 1982).

Nevertheless, fixation rate in the experiments seems to be a little smaller than that ^{15}N natural abundance method had predicted. It is probably because the termites which I used in the experiment were rather developed workers and gained little in body weight during the experiment (**Fig. 8**), as Breznak (1975) reported that fully developed workers fixed much smaller nitrogen than worker larvae did. And reduction in fixing rate due to artificial manipulation may also have occurred although dramatic decrease in fixation rate as reported by Prestwich et al. (1980), Prestwich and Bentley (1981) and Lovelock et al. (1985) was not observed .

However, the result of tracer experiment may be consistent with the estimated contribution of nitrogen fixation by the natural abundance method if nitrogen conservation within a colony is considered. For example, termites store uric acid in the fat body and they may be utilized by a colony member though necrophagy or cannibalism (Slaytor and Chappell 1994).

The result that fixing rate were higher in series A (fed on wood tissue) than in series C (fed on filter paper) showed that nitrogen fixation would not occur inversely proportional to the nitrogen content directly in the experimental condition. The result may also arise from abnormal metabolism caused by the incorporated food, but the very reason is not clear.

To estimate the fractionation factors

In my experiment during 8 and 14 weeks of Ar/O_2-gas experiment, there were no significant difference between Ar/O_2-gas and control. This is mainly because the difference in $\delta^{15}N$ of two sources was so small (wood: -3.5±0.3‰, atmospheric nitrogen: from 0 to –2‰) that the change could not be identified even if it occurred.

In the previous section, $\delta^{15}N$ of mangrove tree in which *N. koshunensis* were sampled was all positive. In this section, however, $\delta^{15}N$ of nesting tree was negative, probably because the former site was a swamp forest, where denitrification is often seen and the latter site was a forest, where precipitation (usually negative in $\delta^{15}N$; Yoneyama 1996) may be an important nitrogen source.

In order to study cumulative contribution of nitrogen fixation in the nitrogen economy of a colony level, it is required to keep larvae, soldiers as well as workers. How is the relationship between nitrogen fixation and trophallaxis? Bentley (1984) concluded that nitrogen fixation of soldiers acted as contributor of nitrogen at colony level, rather than 'parasite' as it seemed to be. It is an interesting aspect that should be tested.

Fractionation factor of digesting wood tissue (Δ_{dig}) would have been given by Ar/O_2 experiment, and fractionation factor of nitrogen fixation (Δ_{fix}) by an experiment fed on filter

paper. But since termites can live without diet for a certain period and their turn over rate is slow, much longer period is required to get significant differences. It is required to use termites which nest in the wood with positive $\delta^{15}N$ value, in estimating the fractionation factors. Rather low level tracer ($\delta^{15}N$ <100‰) and several series with different $\delta^{15}N$ air are required. It is better to incorporate $^{14}N^{15}N$ rather than $^{15}N^{15}N$ for its accuracy in the analysis. In each measurement, $\delta^{15}N$ of termites should be plotted against $\delta^{15}N$ of air. And regression line should be added to both "series A" (feed on nesting wood) and "series C" (feed on filter paper). Zero intercept of $\delta^{15}N_{air}$ of the regression line will show $\delta^{15}N_{wood} + \Delta_{dig} + \Delta_{fix}$ for series A and $\delta^{15}N_{wood} + \Delta_{dig}$ for series C.

4. From wood- to soil-feeding termites: A study in a tropical forest in Cameroon, central Africa

In this chapter, I investigated the stable isotope ratios of termites collected from the large assemblage present in the Mbalmayo Forest Reserve, southern Cameroon. Nitrogen and carbon isotope ratios have been obtained for termite tissues, mound/nest materials and associated substrates (wood, litter and soil) in representative wood-feeding, wood/soil interface feeding and soil-feeding species. The results were consistent with the identifications of ingested materials given by gut content analysis and provided further insights into the feeding ecology of forest termites. A study of litter foragers, especially the Macrotermitinae, was reported in Chapter 6.

4.1 Materials and methods

Sampling sites

Observations and sampling were made in the Mbalmayo Forest Reserve, southern Cameroon (4°N, 13°E; **Fig. 12**). The forest is classified as lowland moist tropical forest in the Holdridge life-zone system (Holdridge et al. 1971). Annual rainfall averages 1520 mm and falls during two wet seasons, March to June, and September to November. Average monthly temperatures fluctuate by only 3°C, from 22.6°C in August to 25.5°C in January. Termite abundance is very high, with up to 10,000 individuals m^{-2} (Eggleton et al. 1995, 1996) and more than 100 species in the assemblage, including representatives of all major trophic groups (Eggleton et al. 1996).

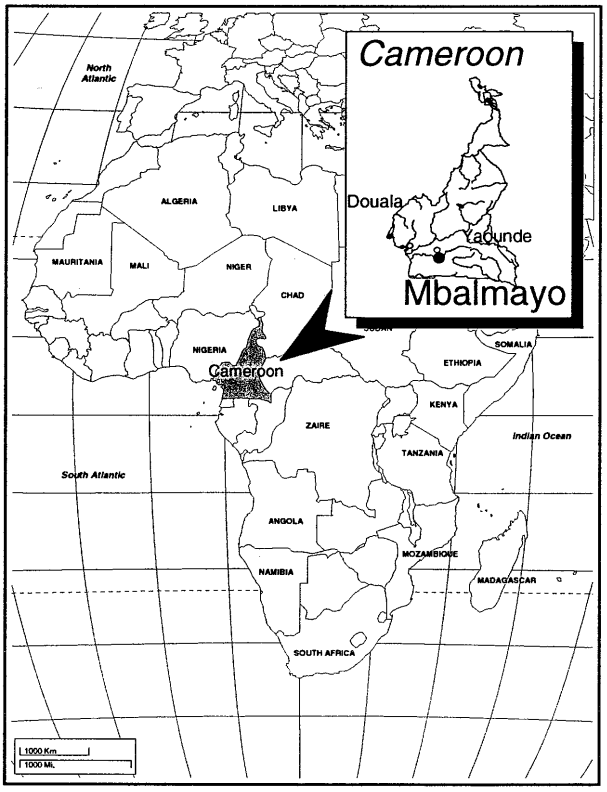


Fig. 12 Sampling site in Cameroon.

Sampling was carried out during July 11–August 7, 1994 in an old growth plantation of *Terminalia ivorensis* ,

adjacent to the Humid Forest Station of the International Institute of Tropical Agriculture (IITA), and in "near primary" forest (terminology of Eggleton et al. 1995) on the Bilik Plateau. The species selected (**Table 7**) were considered representative of the recognized trophic groups and generally amongst those which are common or locally abundant in primary or secondary forest. In all cases termites could be sampled and returned to the laboratory within 1 hour.

Sampling procedure

Wherever possible, termites were collected as worker and soldier castes (*Astalotermes* is soldierless), and as immature forms (developing worker larvae). I collected associated samples as follows: in the case of soil-feeders, soil materials adjacent to the nest, deeper soil (nearly 10 cm depth) and soil at 1 to 2 m distance from the nest were collected. Part of the above ground portion of the mound in *Cubitermes heghi, Cubitermes fungifaber, Astalotermes quietus, Procubitermes arboricola* and *Thoracotermes macrothorax* (all soil-feeders) were also sampled for comparison with the soil material. *Jugositermes tuberculatus* and *Astalotermes* sp. nov. (also soil-feeders) have below-ground colony centers that I could not sample, but individuals or groups can be found in the surface 5 cm of soil, which was assumed to be the dietary material consumed.

Microcerotermes parvus and *Nasutitermes latifrons* are common wood-feeders that nest in dead wood or arboreally and subsist on woody tissue. They are the "intermediate type" of Abe (1987), in that they feed predominantly on the wood within which they nest but

Table 7. Termite species sampled in the Mbalmayo Forest Reserve. Feeding group designations are from Eggleton et al. (1995). All the species listed are higher termites (Family Termitidae).

Species	Number of colonies sampled	Sampling plot[a]	Feeding group[b]	Taxonomic group (subfamily)
Cubitermes heghi	2	OP	S	Termitinae
Cubitermes fungifaber	3	OP	S	Termitinae
Thoracotermes macrothorax	1	NP	S	Termitinae
Procubitermes arboricola	1	OP	S	Termitinae
Astalotermes sp.nov.	1	NP	S	Apicotermitinae
Jugositermes tuberculatus	1	NP	S	Apicotermitinae
Astalotermes quietus	1	OP	S	Apicotermitinae
Termes hospes	2	OP	W	Termitinae
Nasutitermes latifrons	1	OP	W	Nasutitermitinae
Cephalotermes rectangularis	1	OP	W	Termitinae
Microcerotermes parvus	5	OP	W	Termitinae
Acanthotermes acanthothorax[c]	1	OP	W, L (F)	Macrotermitinae

[a] OP:old plantation, NP:near primary forest (*cf.* Eggleton et al. 1995).

[b] S:soil feeder, W:wood feeder, W, L (F):wood and litter feeder (fungus grower).

[c] *A. acanthothorax*, found in the same wood where *M. parvus* (colony 4) nested.

do forage elsewhere. Therefore I collected nesting wood as their diet. Here, I regard *M. parvus* as typical wood-feeder because it feeds relatively undecayed wood, although the extreme "one-piece type" termites, which subsist solely on the nesting wood, predominantly the Kalotermitidae (Abe 1987), are not present in the Mbalmayo Reserve. A fungus growing termite, *Acanthotermes acanthothorax* , which was found in the wood where *M. parvus* nested (nest 4) was also collected. This species is also a wood-feeder.

Termes hospes and *Cephalotermes rectangularis* are "separate type", which forage away from their nest (Abe 1987). The foraging site is usually a decaying tree stump or other large items of dead wood against which the nest is constructed. Hence, I sampled wood that was observed to be eaten, close to each species' nest. *T. hospes* is a wood/soil interface feeder.

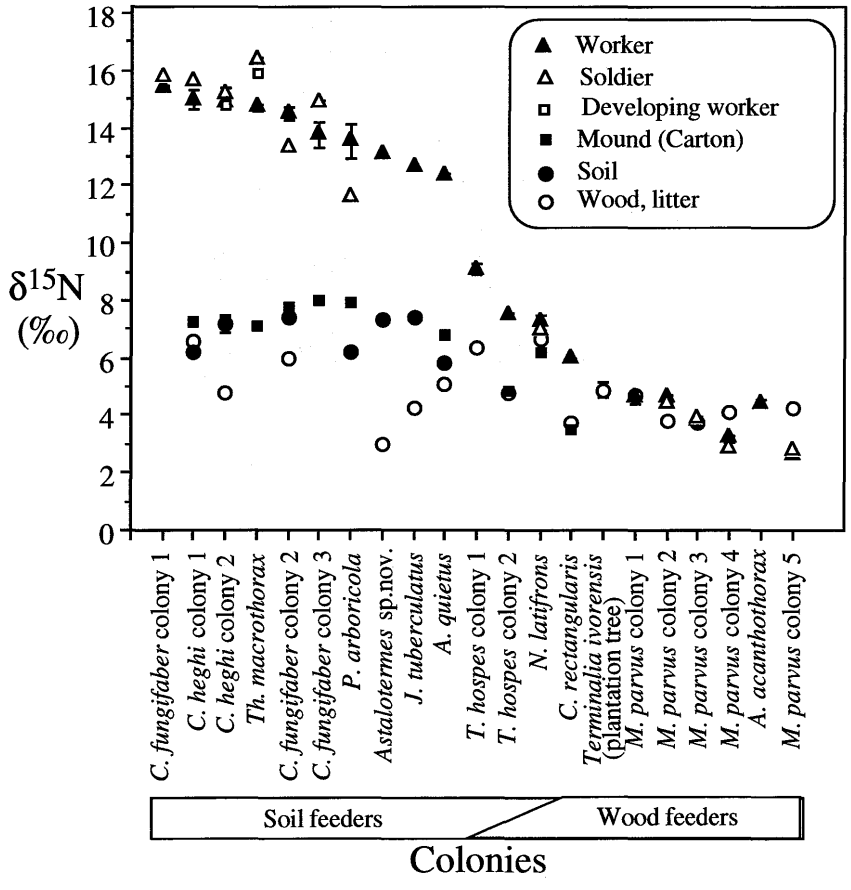


Fig. 13 $\delta^{15}N$ values of tissues (3 castes), mound/nest materials and dietary substances in 10 representative species of termites from the Mbalmayo Forest Reserve. The colonies sampled are shown on the abscissa, with species arranged in descending order (L to R) of $\delta^{15}N$ of workers. Mean and SE are given for worker castes (n= 1 - 3, 1 - 17 workers were used for each measurement), mound material, soil and wood, litter (n= 1- 3), otherwise means only. An additional single value is included for sound wood of *Terminalia ivorensis* , a common plantation tree in the Reserve. *A. acanthothorax* was found in the same dead wood where *M. parvus* (colony 4) nested.

4.2 Results

Nitrogen and carbon isotope ratios among termites

There were differences in $\delta^{15}N$ between species and among castes within the same species. The $\delta^{15}N$ values of soils were higher than those of wood tissue and leaf litter and those of mound material of soil-feeders were higher still, to a maximum value of 8.0‰ **(Fig. 13)**. $\delta^{15}N$ of mound material was 0.2 to 1.7‰ higher than that of soils. Soil-feeder tissues ($\delta^{15}N$ = 12.3–15.4‰) had significantly higher $\delta^{15}N$ values than wood- and litter-feeders, including Macrotermitinae (Kruskal-Wallis ANOVA, $p<0.01$). Amongst soil-feeders the Termitinae (*C. fungifaber* , *C. heghi* , *T. macrothorax* and *P. arboricola*) had significantly higher $\delta^{15}N$ than the Apicotermitinae (*A. quietus* , *A.* sp. nov. and *J. tuberculatus* ; Kruskal-Wallis ANOVA, $p<0.05$).

$\delta^{13}C$ values of termite tissues and dietary substances (wood and soils) are shown in **Fig. 14**. Again, there were differences in $\delta^{13}C$ between species and among castes within the same species. $\delta^{13}C$ values of wood tissue and leaf litter were -29.2 to -25.0‰, those of soils were -27.9 to -27.0‰, and those of mound material of soil-feeders were -28.3 to -26.7‰. $\delta^{13}C$ of

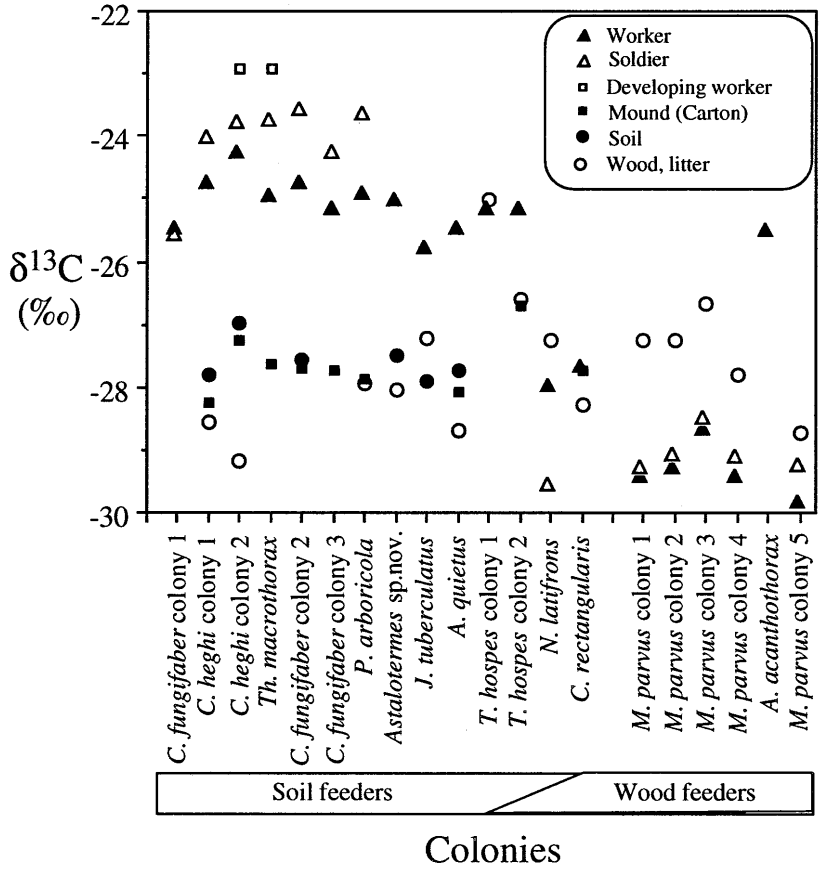


Fig. 14 $\delta^{13}C$ values of termite tissues, mound nest materials and dietary substances. For further explanation, see Fig. 13.

mound material were similar to those of soils. $\delta^{13}C$ values of termites were also different between trophic groups. Soil- and wood/soil interface feeders had significantly higher $\delta^{13}C$ values than wood- and litter-feeders (Kruskal-Wallis ANOVA, $p<0.05$). In this case there was no significant difference between the $\delta^{13}C$ of soil-feeding Apicotermitinae and soil-feeding Termitinae (including *T. hospes*; Kruskal-Wallis ANOVA, $p>0.1$).

$\delta^{15}N$ of workers is plotted against $\delta^{13}C$ of workers in **Fig. 15**. Here, species from *C. fungifaber* to *A. quietus* (inclusive, **Table 7**) are shown representing soil feeders and *M. parvus* is plotted as a typical sound wood-feeder. In the figure, soil-feeding and wood-feeding species cluster separately. Other species (*T. hospes*, *N. latifrons* and *C. rectangularis*) cluster between these groups. Soil-feeders have relatively high values of $\delta^{15}N$ and $\delta^{13}C$, while wood-feeders have relatively low $\delta^{15}N$ and $\delta^{13}C$. The wood/soil interface feeder, *T. hospes*, and the fungus associated *A. acanthothorax* were lower $\delta^{15}N$ but comparable $\delta^{13}C$ values to soil-feeders.

Isotope ratios within the termite body

The workers of *C. heghi* and *C. fungifaber* had similar $\delta^{15}N$ in the P1 (first proctodaeal segment)/P3(main hindgut chamber), head, body and whole worker caste, however the crop (foregut) and rectum (P5 of hindgut) had lower $\delta^{15}N$ values than other regions, although they were higher than those of the soils in which the termites fed **(Fig.16)**. The high $\delta^{15}N$ of termite tissues cannot therefore be explained by selective feeding from a portion or portions of the soil, but must reflect a fractionation occurring as a result of the

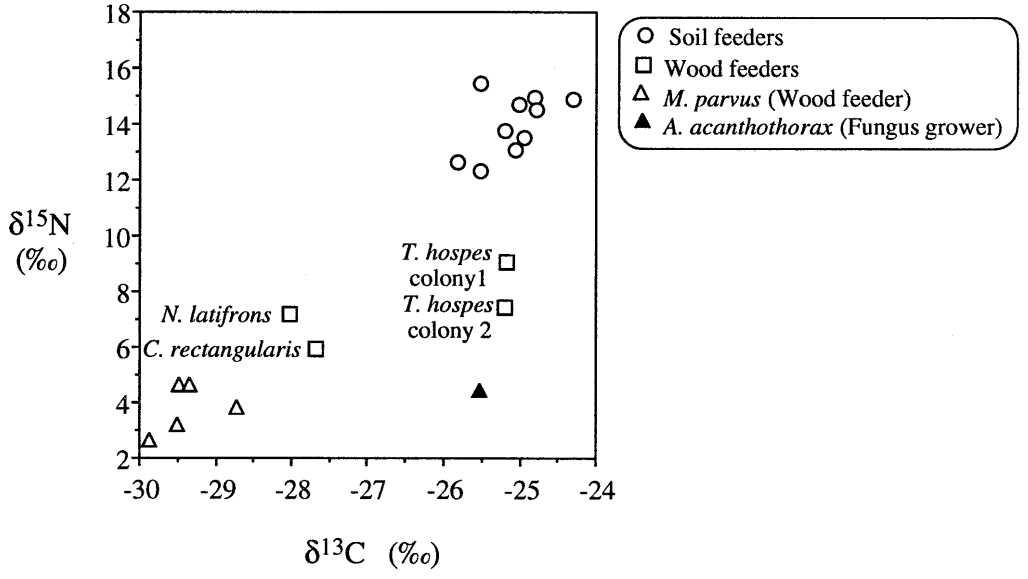


Fig. 15 Plot of mean $\delta^{15}N$ against mean $\delta^{13}C$ for the tissues of worker caste termites. Soil feeding species (colonies of 7 species, open circles) cluster at top right and colonies of the wood-feeding *M. parvus* (open triangles) at lower left. Other species are shown by name.

digestive and assimilative process. The main part of hindgut (P1/P3) is a high pH region (Bignell and Anderson 1980, Bignell and Eggleton 1995, approximate values shown in **Fig. 16**), but it is unclear whether this or a selective assimilation from ingested materials can account for the difference in $\delta^{15}N$ observed.

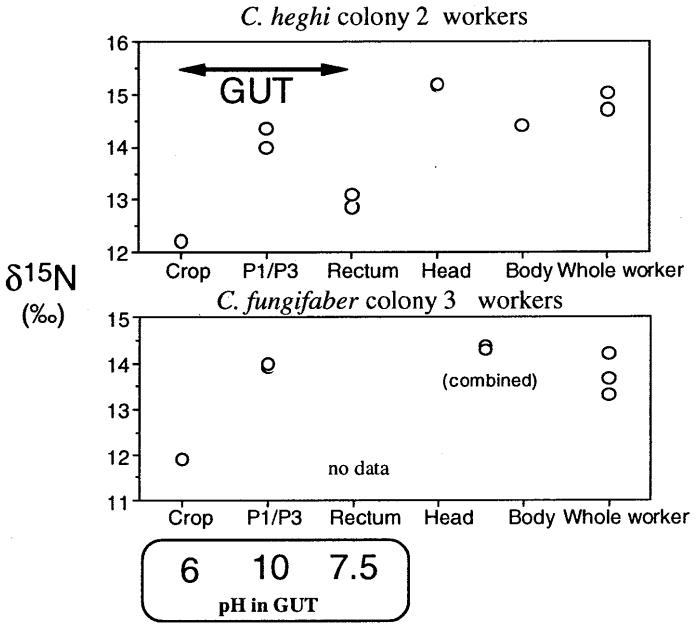


Fig. 16 $\delta^{15}N$ of gut regions (with contents), head capsule and the remaining body (each subsmple was taken from 100-150 individuals) in workers of *C. heghi* and *C. fungifaber*. Gut regions were crop (foregut), P1(first proctodaeal segment)/P3(main hindgut chamber) and rectum (P5 of hindgut). Termites were sampled from colony 2 (*C. heghi*) and colony 3 (*C. fungifaber*). Gut pH (determined by narrow range pH paper) of *C. fungifaber* is shown at the foot of the figure. pH in *C. heghi* was similar.

Carbon and nitrogen content of the diet

Woody tissue has carbon concentrations significantly greater (10- to 20-times) that of soil (Mann-Whitney U-test, $p<0.01$) (**Table 8**). However, there is no significant difference between mound/soil N concentrations and wood N concentrations ($p>0.1$). The carbon/ nitrogen (C/N) ratio of wood ranges from 75 to 247 while that of soil is 9 to 14. Termites contain 6.7–11.0% nitrogen and their C/N ratio is 4.3–6.9 .

4.3 Discussion

Nitrogen stable isotope ratios as an indicator of food preference

The evident repeated evolutionary trends from wood- to soil-feeding forms have been discussed in Noirot (1992) and Bignell (1994). It is suggested that the loss of a protozoan-dominated intestinal flora in higher termites, and their replacement by an exclusively prokaryotic assemblage of gut symbionts, has made possible the diversification of trophic groups to include forms feeding on highly decayed wood and mineral soil fractions in which the organic resource is either humified aromatic organic matter largely derived from lignin, or residual, dispersed cellulose (Grassé and Noirot 1959, Bignell 1994, Breznak and Brune 1994). It is unclear whether any active selection of the many components present is involved during ingestion. Amongst soil-feeding species there exist conspicuous variations in the morphology and pH regimes of the gut, suggesting that the biochemistry of digestion and/or

Table 8. Carbon and nitrogen content of dietary substances.

Species	Colony No.	Material analysed[a]	Carbon content(%)[b]	Nitrogen content(%)[b]	C/N ratio
Cubitermes heghi	1	S	5.8	0.42	12.
	2	S	3.3	0.27	12
		M	3.5	0.33	10
Cubitermes fungifaber	1		N.D.	N.D.	
	2		N.D.	N.D.	
	3	M	3.4	0.34	14
Thoracotermes macrothorax		M	5.7	0.50	12
Procubitermes arboricola		S	2.7	0.22	12
		M	2.7	0.33	9
Astalotermes sp.nov.			N.D.	N.D.	
Jugositermes tuberculatus			N.D.	N.D.	
Astalotermes quietus		M	8.3	0.62	13
Termes hospes	1	W	49.5	0.27	181
	2	W	49.0	0.44	110
Nasutitermes latifrons		W	50.9	0.54	94
Cephalotermes rectangularis		W	47.7	0.43	110
Microcerotermes parvus	1	W	48.0	0.41	117
	2	W	45.9	0.32	146
	3	W	48.4	0.20	247
	4	W	45.6	0.61	75
	5	W	46.5	0.24	191
Acanthotermes acanthothorax		W(F)	45.6[c]	0.61[c]	75

[a] S, soil adjacent to the mound nest of soil feeder; M, mound of soil feeder; W, wood fed by wood feeder, W(F), wood consumed by fungus grower.

[b] % by weight; mean value (n=3) measured by a CHN corder, Fisons type EA1108.

[c] the same wood in which *M. parvus* (colony 4) nested.

the intestinal microflora may vary (Bignell and Eggleton 1995).

In stable isotope studies of nutrient assimilation, $\Delta\delta^{15}N$ averages +3.4‰ (DeNiro and Epstein 1981, Minagawa and Wada 1984). However, values of $\Delta\delta^{15}N$ for soil-feeding termites were higher than this, while lower in wood-feeders (**Table 9**). The $\Delta\delta^{15}N$ values of *T. hospes*, *N. latifrons* and *C. rectangularis* were intermediate. The $\Delta\delta^{15}N$ of soil-feeders (maximum value 8.8 ‰ in *C. heghi*) is consistent with the large apparent isotope effect observed in a deposit-feeder, *Neanthes japonica* (Polychaeta, Annelida), in Otsuchi Bay, Japan ($\Delta\delta^{15}N = 5\pm1$‰; Kikuchi and Wada 1996). The large isotope effect may therefore be characteristic of the digestion of organo-mineral complexes and could be explained as follows: 1) selective assimilation of bacteria or bacterially-mediated compounds from the diet may cause a large fractionation, due to microbial immobilization of nitrogen or denitrification at microsites (Kikuchi and Wada 1996). 2) It may reflect the degradation of tannin-protein complexes in the termite gut, a possible digestive mechanism in soil-feeding

Table 9. Comparison of $\delta^{15}N$ and $\Delta\delta^{15}N$ (isotope effect; $\Delta\delta^{15}N=\Delta\delta^{15}N_{termite} - \Delta\delta^{15}N_{diet}$) in termite species along a hypothetical humification gradient (soil to wood).

Species	Feeding group[a]	$\delta^{15}N$ range (‰) (number of the nests studied)	$\Delta\delta^{15}N$ range (‰) (number of the nests studied)
Cubitermes heghi	S	14.9– 15.0 (2)	7.7– 8.8 (2)
Cubitermes fungifaber	S	13.7– 15.4(3)	5.8– 8.6 (3)
Thoracotermes macrothorax	S	14.7	7.6
Procubitermes arboricola	S	13.5	7.3
Astalotermes sp.nov.	S	13.1	5.7
Jugositermes tuberculatus	S	12.6	5.3
Astalotermes quietus	S	12.3	6.5
Termes hospes	W	7.5– 9.1 (2)	2.7 (2)
Nasutitermes latifrons	W	7.2	0.5
Cephalotermes rectangularis	W	5.9	2.2
Microcerotermes parvus	W	2.6– 4.6(5)	-1.6– 0.8 (5)
Acanthotermes acanthothorax	W L (F)	4.4	0.3

[a] S, soil feeder; W, wood feeder; W L (F), wood and litter feeder (fungus grower).

forms (Bignell 1994). 3) Termites may prefer silt and clay fractions to larger particles (Anderson and Wood 1984), and since $\delta^{15}N$ in forest soil is negatively correlated with particle size, reflecting a gradual enrichment of $\delta^{15}N$ in the decomposition process (Tiessen et al. 1984, Nadelhoffer and Fry 1994), the effect results from such selection.

Table 9 shows the termite species examined in this study listed in descending order of $\delta^{15}N$. The rank order of $\delta^{15}N$ correlates strongly with the feeding order of species along a hypothetical humification gradient shown by gut content analysis (Sleaford et al. 1996; Spearman's rank correlation, $p<0.01$). *T. hospes* and *C. rectangularis* cluster between the other two groups of typical wood-feeders and soil-feeders, which may reflect the fact that they feed at the wood/soil interface or on very rotten wood . This finding suggests that $\delta^{15}N$ of termite tissues and the isotope effect ($\Delta\delta^{15}N$) could be a valuable tool in specifying the diet of species for which other information was not available (for example subterranean forms).

Inquiline species in the termite mound

$\delta^{15}N$ can be used as food web indicators for animals (Minagawa and Wada 1984). $\delta^{15}N$ of *Microtermes* sp. and that of an unidentified species of ant, which are inquilines (i.e. animals which live in nests build by other species) of *C. fungifaber* (colony 3), were 4.6‰ and 9.5‰ respectively, while $\delta^{15}N$ of the host was 13.7‰ (**Fig. 13**, but inquiline species were not shown in the figure). The result suggests that these inquiline species were independent of the host termites assuming $\Delta\delta^{15}N$ of 3.4‰ (Wada et al. 1991) .

Nitrogen fixation in soil-feeders and wood-feeders

% N_{dfa} of *Microcerotermes parvus* was calculated using the assumption of $\Delta_{dig} > 0$ ‰ and -2 ‰$< \Delta_{fix} < 0$ ‰ (Tayasu et al. 1994) of the equation (6). Among the five colonies of a wood-feeder, *M. parvus* , only two colonies had estimated values of $\%N_{dfa} > 15\%$ (colonies 4 and 5) . Although more extensive experiments to estimate isotope effects are required, nitrogen fixation may be of minor importance for *M. parvus* (given the above assumptions). The suggestion that nitrogen fixation contributes less in *M. parvus* than in *N. koshunensis* raises many questions. The former is a higher termite (Termitidae), "intermediate type", whereas the latter is a lower termite (Kalotermitidae), "one-piece type" (terminologies of Abe, 1987). Furthermore, nitrogen content of the diet ranges 0.20–0.61% in *M. parvus* **(Table 8)** but from 0.07–0.31% in *N. koshunensis* **(Table 15)** and are significantly different (Mann-Whitney U-test, $p<0.05$).

The high $\delta^{15}N$ of soil-feeders indicates that nitrogen fixation is negligible in these species ($\delta^{15}N_{atmosphere} = 0$) . In wood-feeding termites, the C/N ratio of the diet is so high that a balance can be achieved only by increasing nitrogen-intake (from another source) or selectively eliminating carbon (Higashi et al. 1992). Soil-feeding termites, however, appear to have no need to acquire additional nitrogen, as the C/N ratio of the material they ingest is much more favorable **(Table 8)**. Indeed, if the dispersed cellulosic substances present in soil are the principal substrates digested in the guts of soil-feeders, as suggested by Grassé and Noirot (1959), the yield of energy may not be sufficient to meet the high ATP cost of nitrogen fixation.

Carbon isotope effect in termites

Carbon isotope ratios reflect two elements of termite biology: 1) the carbon source consumed and 2) the intestinal interactions between the termite and its gut microbes, particularly the balance between methanogenesis and acetogenesis. Previous stable isotope studies of animal digestive assimilation suggest that $\Delta\delta^{13}C$ is small ($<\pm 1$‰) in most cases (DeNiro and Epstein 1978). However, $\Delta\delta^{13}C$ of *A. acanthothorax* (Macrotermitinae) was 2.3‰ whereas that of *M. parvus* (nest 4) was -1.7‰. The positive isotope effect of the fungus grower is probably explained by the decomposition processes taking place in the fungus garden. This is supported by the observation that in minor workers of *Macrotermes muelleri* (another fungus-associated species occurring in the Mbalmayo Forest Reserve), the termite tissues were 4.6‰ enriched compared with the diet of grass and leaf litter (Chapter 7). The high $\delta^{13}C$ enrichment in soil-feeders may be consistent with the following results. Martin et al. (1992) found that a geophagous earthworm in a tropical savanna had 4‰ greater $\delta^{13}C$ than that of soil organic matter. Microbial $\delta^{13}C$ were averaged 3.5‰ higher than that of

amino acids on which they were grown (Macko and Estep 1984). Similarly, Ryan et al. (1995) reported that soil microbial biomass had 2.4‰ higher $\delta^{13}C$ than that of bulk soil organic matter in an agricultural system. Nadelhoffer and Fry (1988) found $\delta^{13}C$ of bulk soil organic matter increased by up to 0.5‰ over a 600-day laboratory incubation.

There is another possibility of mechanisms that may account for abnormal isotope effect. Anaerobic acetogenesis from CO_2/H_2 in the gut possibly lowers $\delta^{13}C$ of termites (Sugimoto et al. in prep.). Brauman et al. (1992) pointed out that acetogenesis is favored in wood-feeders whereas methanogenesis is favored in fungus growers and soil-feeders. Bignell et al. (1997) observed a similar pattern within the Cameroon assemblage; the rate of methane production in *M. parvus* was very low (<0.05 μmol g^{-1} h^{-1}). $\Delta\delta^{13}C$ of this wood-feeding species was -2.2 to -1.1‰ **(Fig. 14)**, which suggests that acetogenesis is predominant. In soil feeders, on the other hand, $\Delta\delta^{13}C$ is positive and in the region of 2.0 to 3.0‰. This is consistent with their much higher rates of methane production (for example *C. fungifaber*, *C. heghi*, *T. macrothorax*, *J. tuberculatus* and *A. quietus* range 0.20–0.30 μmol g^{-1} h^{-1}; see Bignell et al., 1997).

In conclusion, stable isotope analyses confirm the diversity of termite feeding habits and suggest that differences exist in some of the fundamental mechanisms of digestion and nutrient assimilation between trophic groups.

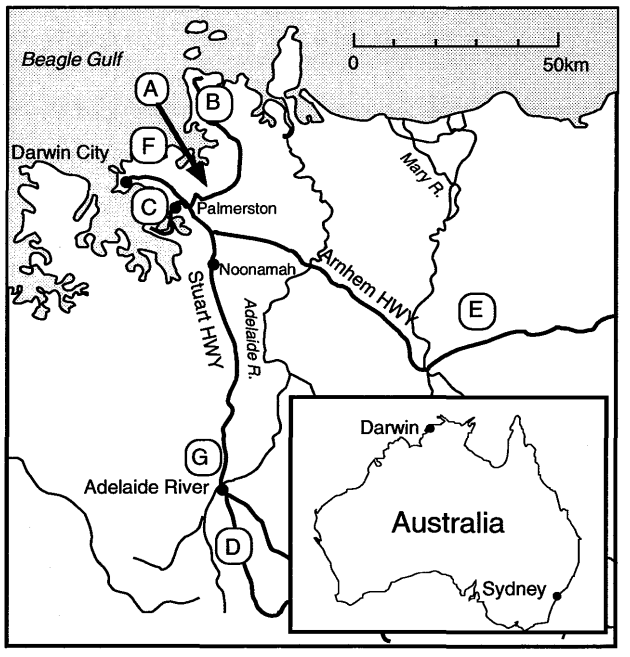
5. DIVERSIFICATION AND EVOLUTION OF HUMIVORE IN *TERMES-CAPRITERMES* BRANCH OF THE SUBFAMILY TERMITINAE IN NORTHERN AUSTRALIA

In the previous chapter, I indicated that nitrogen stable isotope ratio of termites was heuristic indicator of a functional position in the humification process. In this chapter, I presented stable isotope analysis of Australian termites, especially the *Termes-Capritermes* branch species, and compared with molecular phylogenetic work (Inoue et al. in prep.) in order to study the evolution and diversification from wood-feeding to soil-feeding in Australia. Results were compared with data of termite species from Cameroon.

5.1 Sampling

Samplings and observations were made in the suburbs of Darwin City (12°24'S, 130°53'E), the capital of Northern Territory (NT) in Australia **(Fig.17)**. Average temperature is 27.7°C, and annual rainfall averages 1705 mm and most rainfall occurs during the wet season, December to March.

Sampling was carried out during February 28 –March 21, 1996. I collected 47 colonies of 22 species (four families, including two existing subfamilies of the Termitidae) **(Table 10)**. Termites, wood, soil and mound material were prepared for stable isotope ratios as described in Chapter 2.



A: Howard Springs-Gunn Point Road (Monsoon forest)
B: Prison reserve (Monsoon forest)
C: Channel Island (Mangrove forest)
D: Historic HWY-Adelaide River (Woodland)
E: Point Stuart-Arnhem HWY (Woodland)
F: Howards Peninsula (Monsoon forest)
G: Stuart HWY-Adelaide River (Woodland)

Fig. 17 Sampling sites in Darwin

5.2 Results

An outline of the C/N components of nest and mound material

Nesting habits are shown in **Table**

Table 10 Termite species sampled in Darwin region.

Species	Number of colonies sampled	Sampling plot*	Nesting habit¶	Taxonomic group (Family)#
– –Lower termites				
Mastotermes darwiniensis	2	A, B	W	Mastotermitidae
Cryptotermes secundus	5	C	W	Kalotermitidae
Coptotermes acinaciformis	1	B	M	Rhinotermitidae
Heterotermes vagus	1	B	W	Rhinotermitidae
Schedorhinotermes actuosus	1	B	M	Rhinotermitidae
Schedorhinotermes breinli	1	B	W	Rhinotermitidae
– –Higher termites				
Microcerotermes boreus	1	A	M	Termitidae (T)
Microcerotermes nervosus	3	A, B, D	M	Termitidae (T)
Amitermes arboreus	2	A, D	M	Termitidae (T)
Amitermes laurensis	1	E	M	Termitidae (T)
Amitermes perelegans	2	B	M	Termitidae (T)
Amitermes subtilis	1	A	M	Termitidae (T)
Xylochomitermes melvillensis	4	A, B, E, F	W	Termitidae (T)
Ephelotermes melachoma	3	A(2), D	M	Termitidae (T)
Cristatitermes carinatus	1	B	M	Termitidae (T)
Cristatitermes froggatti	3	B(2), D	M	Termitidae (T)
Hapsidotermes orbus	1	D	M	Termitidae (T)
Lophotermes quadratus	1	D	M	Termitidae (T)
Lophotermes septentrionalis	1	A	M	Termitidae (T)
Macrognathotermes sunteri	10	A(2) ,B(5) D(2), E(1)	M	Termitidae (T)
Nasutitermes triodiae	1	G	M	Termitidae (N)
Tumulitermes comatus	1	B	M	Termitidae (N)

* A: Howard Springs-Gunn Point Road (Monsoon forest), B: Prison reserve (Monsoon forest), C: Channel Island (Mangrove forest), D: Historic HWY-Adelaide River (Woodland), E: Point Stuart-Arnhem HWY (Woodland), F: Howards Peninsula (Monsoon forest), G: Stuart HWY-Adelaide River (Woodland), where colony numbers were enclosed in parenthesis.

¶ W: in wood, M: in the mound

Subfamilies were added in parenthesis for Termitidae. T:Termitinae, N: Nasutitermitinae.

10. In the table, nesting habits were divided into two categories; nesting in the wood tissue or in a mound. The latter includes the nesting in the mound built by the colony itself and by the other colonies (inquiline or take-over species).

Carbon and nitrogen content of woody material that would be regarded as food ranged 46.4-55.4% and 0.11-0.51%, respectively, and C/N ratio were 101-492 (**Table 11**). Those of mound material ranged 2.2-21.6% and 0.10-0.51%, respectively, and C/N ratio was 20-50. C/N of soil was 20-24 (n=2, data are not shown in the table).

$\delta^{13}C$ and $\delta^{15}N$ of termites

$\delta^{13}C$ of workers ranged from -27.3‰ to -12.8‰ and $\delta^{15}N$ ranged from -1.5‰ to 8.2‰

Table 11 Carbon and nitrogen content of wood and termite mound, together with $\delta^{15}N$ of mound material. Number of colonies studied were written as n and each value was based on triplicates.

Species	Wood				Mound				
	n	C (%)	N (%)	C/N	n	C (%)	N (%)	C/N	$\delta^{15}N$
– Lower termites									
Mastotermes darwiniensis	1	51.5	0.18	288	–	–	–	–	–
Cryptotermes secundus	5	46.4-47.5	0.17-0.40	120-282	–	–	–	–	–
Heterotermes vagus	1	55.4	0.11	492	–	–	–	–	–
Schedorhinotermes actuosus	1	51.1	0.12	420	1	5.3	0.16	32	1.6
Schedorhinotermes breinli	1	49.8	0.25	201	–	–	–	–	–
– Higher termites									
Microcerotermes nervosus	1	49.1	0.29	169	3	5.1-15.6	0.15-0.40	29-39	-0.6-2.7
Amitermes arboreus	1	50.4	0.27	184	1	21.6	0.43	50	-0.5
Amitermes laurensis	–	–	–	–	1	2.2	0.10	22	2.0
Amitermes perelegans	–	–	–	–	1	8.1	0.32	26	2.5
Amitermes subtilis	–	–	–	–	1	2.3	0.12	20	4.3
Xylochomitermes melvillensis	4	48.3-52.2	0.16-0.51	101-314	–	–	–	–	–
Ephelotermes melachoma	2	49.9-50.6	0.16-0.19	267-318	3	10.9-16.0	0.40-0.51	23-34	0.0-1.6
Cristatitermes froggatti	1	52.2	0.22	239	3	4.3-7.6	0.19-0.29	22-27	1.5-2.0
Hapsidotermes orbus	–	–	–	–	1	3.7	0.18	21	0.7
Lophotermes quadratus	–	–	–	–	1	7.7	0.31	25	0.5
Lophotermes septentrionalis	–	–	–	–	1	6.7	0.24	28	0.7
Macrognathotermes sunteri	2	48.6-51.6	0.27	181-192	10	6.1-10.6	0.23-0.36	24-37	-0.5-1.9
Nasutitermes triodiae	–	–	–	–	1	2.2	0.10	22	2.1
Tumulitermes comatus	1	49.1	0.22	221	–	–	–	–	–

(Fig. 18).

Ten colonies of *Macrognathotermes sunteri,* which made epigeal nest, were sampled at four plots in Darwin **(Table 10)**. $\delta^{13}C$ and $\delta^{15}N$ of these mound materials were not significantly different among the plots (Kruskal-Wallis, $p>0.2$ for $\delta^{13}C$ and $\delta^{15}N$; **Table 11**). $\delta^{13}C$ and $\delta^{15}N$ of the mound material of *Microcerotermes nervosus, Cristatitermes froggatti, Ephelotermes melachoma* and *Macrognathotermes sunteri* were, also, not significantly different among species (ANOVA test, $p>0.9$ for $\delta^{13}C$ and $p>0.5$ for $\delta^{15}N$; **Table 11**). These data dismissed the hypothesis that the difference was due to the isotopic variation among sampling plots or the specific preference of $\delta^{13}C$ and $\delta^{15}N$ in soil.

On the other hand, $\delta^{13}C_{worker}$ was significantly lower in *Cryptotermes secundus, Microcerotermes nervosus* and *Xylochomitermes melvillensis* than in *Ephelotermes melachoma, Cristatitermes froggatti* and *Macrognathotermes sunteri* (ANOVA, Fisher Post Hoc test, $p<0.001$). Also, $\delta^{15}N_{worker}$ was significantly different and enriched along with *C. secundus, M. nervosus* then *X. melvillensis, E. melachoma* to the maximum of *C. froggatti, M. sunteri* (ANOVA, Fisher Post Hoc test, $p<0.001$) **(Fig. 18)**.

Variation in $\delta^{15}N$ was also observed in the genus *Amitermes*, which is most diverse in

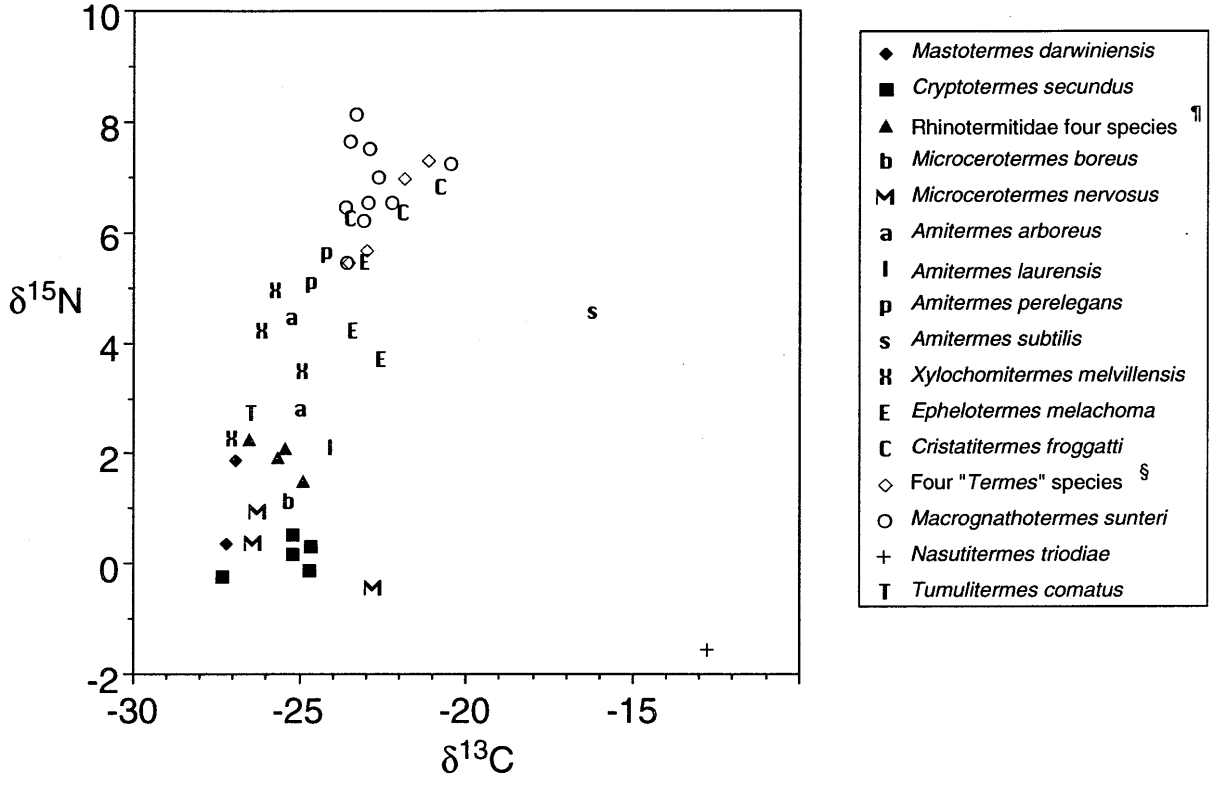


Fig. 18 $\delta^{13}C$ and $\delta^{15}N$ of worker termites at Darwin region. Plots were mean value of triplicates of measurements, where several individuals were used for each measurement.
¶Rhinotermitidae four species denote *Coptotermes acinaciformis, Heterotermes vagus, Schedorhinotermes actuosus* and *S. breinli.*
§ Four "Termes" species denote *Termes-Capritermes* species (*Cristatitermes carinatus, Hapsidotermes orbus, Lophotermes quadratus* and *Lophotermes septentrionalis*)

Australia (Miller 1994a). $\delta^{15}N_{worker}$ was enriched in a sequence from *A. laurensis* (2.2‰), *A. arboreous* (2.9‰ and 4.5‰) to *A. perelegans* (5.2‰ and 5.7‰).

$\delta^{13}C$ of *Amitermes subtilis* (-16.2‰) and that of *Neotermes triodiae* (-12.8‰) were distinguished from others.

5.3 Discussion

From wood- to Soil-feeding forms of the subfamily Termitinae in Australia

As discussed in Chapter 4, the feeding habit from wood to soil can be identified by $\delta^{15}N$ of termite body or isotope effect: $\Delta\delta^{15}N = \delta^{15}N_{termite} - \delta^{15}N_{diet}$. However, it is not necessary to use $\Delta\delta^{15}N$ here because $\delta^{15}N$ of wood, soil and mound material were not significantly different among sampling sites nor species. $\delta^{15}N$ values suggest that the dietary preferences along with the humification gradient from wood- to soil-feeding are *C. secundus, M. nervosus* then *X. melvillensis, E. melachoma* to the most humivorous of *C. froggatti, M.*

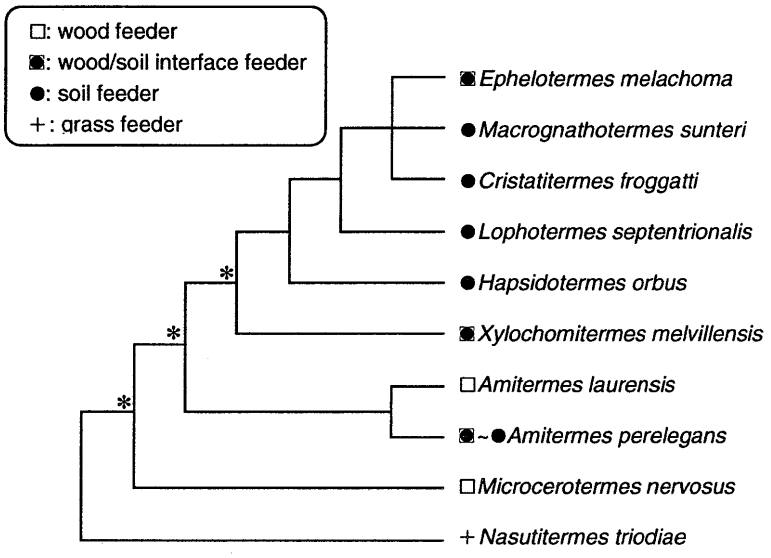


Fig. 19 Molecular phylogenetic tree of some termite species in Darwin, where asterisk mark denotes significant difference (Molecular phylogenetic tree is based on Inoue et al. in prep.).

sunteri. The sequence may be referred to as wood-feeding, wood/soil interface feeding and soil-feeding, respectively, as described in Chapter 4. In a similar way, the classification may be applicable to other species. $\delta^{15}N$ values of four other species of *Termes-Capritermes* branch (*Cristatitermes carinatus, Hapsidotermes orbus, Lophotermes quadratus* and *L. septentrionalis*) were in the soil-feeding region of $\delta^{15}N$ **(Fig. 18)**, although these species were not statistically testable.

Among eight of *Termes-Capritermes* branch species, only two species (*X. melvillensis* and *E. melachoma*) were discernible as wood/soil interface feeders, and remaining six species were soil-feeders. Whereas, Inoue et al. (in prep.) demonstrated that *X. melvillensis* is a basal clade of *Termes-Capritermes* branch species **(Fig. 19)**. The result suggests that wood/soil interface feeding originated when *Termes-Capritermes* species branched. *Termes-Capritermes* branch species probably raised their $\delta^{15}N$ values as they evolved into soil-feeding.

Miller (1991) stated that there were several *Amitermes* species of soil-feeders, two of which were *A. perelegans* and *A. arboreous* on the basis of imago/worker mandibles (Miller 1994a). If the sequence of $\delta^{15}N$ of *Termes-Capritermes* branch species is applicable to *Amitermes* spp., *A. perelegans* and *A. arboreous* ranged from wood/soil interface feeder to soil-feeder. *A. subtilis* had rather high $\delta^{15}N_{worker}$ ($\delta^{15}N$ =4.6‰), but $\Delta\delta^{15}N$ must be less since the mound material had the highest $\delta^{15}N$ of all the nests studied here ($\delta^{15}N$ =4.3‰). *A. subtilis,* whose $\delta^{13}C_{worker}$ (-16.2‰) was quite different from that of mound material (-22.9‰), probably digested both C3 litter and C4 grass in the C3 dominant vegetation. This is

contrastive to *N. triodiae*, which harvested C4 grass in C4 dominant vegetation, for $\delta^{13}C_{worker}$ (-12.8‰) of *N. triodiae* was similar to that of mound material (-14.2‰).

Nitrogen fixation

$\%N_{dfa}$ of *Cryptotermes secundus*, which nested in a wood and consumed the wood tissue, was calculated using the assumptions of $\Delta_{dig} > 0$ ‰ and -2 ‰$< \Delta_{fix} < 0$ ‰ in equation (6) (Tayasu et al. 1994). The results of $\% N_{dfa}$ were at least 48, 45, 15, 31, and 39% for colonies of No. 1 to 5 (**Table A1**, in Appendix section), showing that nitrogen fixation was important in nitrogen economy of *C. secundus*. The contribution of nitrogen fixation in other wood-feeding termites such as *Microcerotermes* spp. could not be estimated because $\delta^{15}N$ of the food sources had not been determined in the natural condition. The high $\delta^{15}N$ of soil-feeders (**Fig. 18**) indicates that nitrogen fixation was negligible in these species .

5.4 Comparison with soil-feeding species in Cameroon

Carbon and nitrogen content of woody material in Cameroon were 45.6-50.9% and 0.27-0.61%, respectively, and C/N ratio was from 75 to 247. Carbon and nitrogen content of mound material of soil feeders in Cameroon were 2.7-8.3% and 0.33-0.62%, respectively, and C/N ratio was 9 to 14 (**Table 8**). C/N ratio of Australian mound material (**Table 11**: mainly 20–30) was significantly higher than that of Cameroon. According to Inoue et al. (in prep.), there were significant cellulase activities in Australian soil-feeding termites, which suggests that cellulose serves as a carbon source. Whereas, soil feeding termites in Africa had a very low level of cellulase, far from that of xylophagous species (Rouland et al. 1986). The results may be explained by the finding that C/N of soil organic matter was higher in Australia than in Cameroon.

M. sunteri showed the most typical soil-feeding $\delta^{15}N$ in Darwin. The difference in $\delta^{15}N$ between termites and their diet ($\Delta\delta^{15}N$) was estimated to be 4.3-7.0‰, using $\delta^{15}N$ of the mound material instead of their diet, for it is difficult to identify. On the other hand, $\Delta\delta^{15}N$ of typical soil-feeders (calculated in the same way) in Cameroon (*Cubitermes heghi, Cubitermes fungifaber* and *Thoracotermes macrothorax*) was 5.8-7.7‰. These values were not significantly different from those of *M. sunteri* (Mann-Whitney U-test, $p>0.05$).

$\delta^{13}C$ and $\delta^{15}N$ of mound material were both significantly different (Mann-Whitney U-test, $p<0.0001$) between Darwin (Australia, **Table 11**: Tayasu et al. in prep.) and Mbalmayo (Cameroon, **Table 8**: Tayasu et al. 1997); -25.7 ± 0.8‰(SD) and 1.1 ± 1.1‰, and -27.8 ± 0.3‰ and 7.4 ± 0.4‰ for $\delta^{13}C$ and $\delta^{15}N$ at Darwin and Mbalmayo, respectively. To compare the variation of $\delta^{15}N$ between Darwin and Mbalmayo, $\delta^{15}N$ values of typical termite species were shown in **Fig. 20**. Both axis were adjusted together to the average $\delta^{15}N$ of the termite nest: 1.1‰ for Darwin and 7.4‰ for Cameroon. Nutritional niche may be characterized by

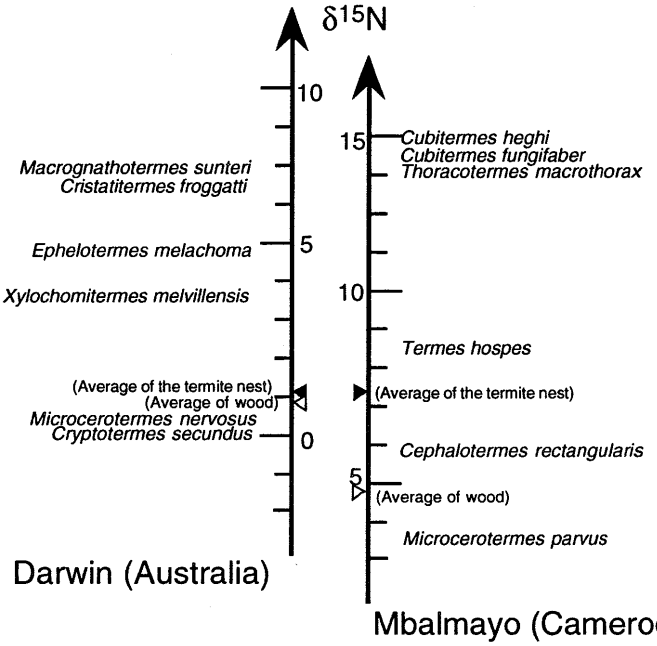


Fig. 20 Comparison of typical termite fauna between Darwin (Australia, Tayasu et al. in prep.) and Mbalmayo (Cameroon, Tayasu et al. 1997). Both axis were adjusted together to the average $\delta^{15}N$ of the termite nest: 1.1‰ for Darwin and 7.4‰ for Cameroon. Average $\delta^{15}N$ of wood tissue (0.8±1.0‰ for Darwin and 4.8±1.1‰ for Cameroon) were added to both Darwin and Cameroon.

the following categories: wood-feeding, wood/soil interface feeding and soil-feeding, as $\delta^{15}N$ varies from low to high. Compared between Darwin and Cameroon, relative $\delta^{15}N$ variation of termites were similar, although the groups of termites were different (**Fig.20**). The wood/soil interface feeders were *Termes-Capritermes* group in both Australia and Cameroon, whereas soil-feeders were *Termes-Capritermes* branch in Australia but *Cubitermes* group in Cameroon. This pattern may have been caused by biogeographical and palaeoecological reasons.

The result confirmed that soil-feeding evolved several times both in Australia and Africa, but from different phylogenetic groups.

6. TWO SHORT NOTES OF $\delta^{13}C$–$\delta^{15}N$ NATURAL ABUNDANCE METHOD

In this chapter, I presented two short notes of the study of termites by using stable isotope techniques. First topic is fungus growing termites in Africa and Asia, and second is wood-feeding and grass-harvesting termites in Australia.

6.1 Fungus growing termites in Cameroon, Thailand and Côte d'Ivore

The subfamily Macrotermitinae (Termitidae, Isoptera) has an ability to access to a range of food materials; dead plant material, dead leaves, branches and grasses, and plays an important role especially in savanna ecosystems. For example, 90% of woody litter was removed by fungus growing termites, *Odontotermes*, *Microtermes* and *Synacanthotermes* in Kenya (Buxton 1981). In Nigeria, 23% of annual litter production was consumed by termites, mainly the Macrotermitinae (Collins 1983). The prosperity lies in the exosymbiotic fungi *Termitomyces* spp., which decompose dead plant materials collected by termites.

In this chapter, I reported carbon and nitrogen isotope ratios of termites, fungal comb and food sources of the Macrotermitinae in Cameroon, Thailand and Côte d'Ivore, and discussed the possible mechanisms that may cause such patterns.

6.1.1 Sampling

Sampling sites

Sampling in Cameroon was carried out during July 11–August 7, 1994 in the "old plantation" area (in the same sampling site as in Chapter 4) of Mbalmayo Forest Reserve. I selected 6 fungus growing species, *Macrotermes muelleri, Macrotermes subhyalinus, Protermes prorepens, Pseudacanthotermes militaris, Acanthotermes acanthothorax* and *Microtermes* sp.. The former three species build conspicuous nests on the ground, while the latter three species make subterranean nests.

Sampling in Thailand was carried out in October 1993 in Kanchanburi, Thailand by Dr. Abe. Average temperature is 28.4°C. Annual rainfall is 1492mm and falls in May to October. A fungus growing termite *Macrotermes annandalei*, which is one of the most important species in the region, were sampled with associated fungus comb.

Sampling in Côte d'Ivore was carried out in Abidjan, Côte d'Ivoire by Dr. Veivers. Average temperature is 26.4°C, and annual rainfall is 1933mm and falls in May to July. *Macrotermes bellicosus* was sampled.

Sampling procedure

Termites were collected and categorized in castes. Fungal nodules were picked up using forceps. Three types of fungal comb could be differentiated in the mound, namely fresh, ripe and old comb (Badertscher et al. 1983). Fresh comb material, which were the primary faeces excreted by young workers, consisted of freshly collected grass as well as conidia from a fungus belonging to the genus *Termitomyces* which is used as a fungal inoculum (Leuthold et al. 1989). Ripe fungal comb consisted of residual plant material and a well-developed mycelium producing aggregated aerial conidiophores (synnemata) which appeared as white spherical bodies (nodules) on the top of the fungal surface. Old fungal comb material was with a reduced density of mycelium and no nodules as these have been eaten by the young workers or have regressed (Martin and Martin 1987). Food material stored in the nest were also sampled. "Food" materials were picked up near the mound of termite nest. Workers of the termites were categorized into four; the foragers are the old major and minor workers (more than 30 days old) with the young major and minor workers (less than 30 days old) involved in processing the forage, construction of the fungal comb and feeding the dependent castes including the larvae and the minor soldiers in *M. muelleri, M. subhyalinus* and *M. bellicosus*. Old and young worker castes for *M. annandalei* was not distinguished.

6.1.2 Results

Nitrogen content of the food and the fungal comb

Nitrogen content of the food and the fungal comb were shown in **Table 12**, which was measured by the Kjeldahl method by Dr Veivers. Nitrogen content was gradually enriched from food store to the fungal comb, with the highest value in the ripe comb.

SI ratio of termites and fungal comb

Fig. 21 shows the $\delta^{13}C$ and $\delta^{15}N$ values of termites, food, food store and fungal nodule in Mbalmayo Forest Reserve in

Table 12 Nirtogen content of the fungal comb and associated materials by weight %.[#]

	Food	Food store	Fungal comb			Worker Termites				Fungal nodule
			Fresh	Ripe	Old	Young minor	Old minor	Young major	Old major	
Macrotermes bellicosus	0.35	0.66	1.26	1.67	1.36	10.90	12.00	7.78	10.90	7.87
Macrotermes muelleri	2.09	1.18	1.70	1.64	1.87	13.68	11.36	11.78	N.D.	4.72
Macrotermes subhyalinus	0.84	0.70	1.48	1.60	0.63	8.32	N.D.	N.D.	N.D.	9.03

N.D. : No data

[#]Measured by Dr P.C. Veivers.

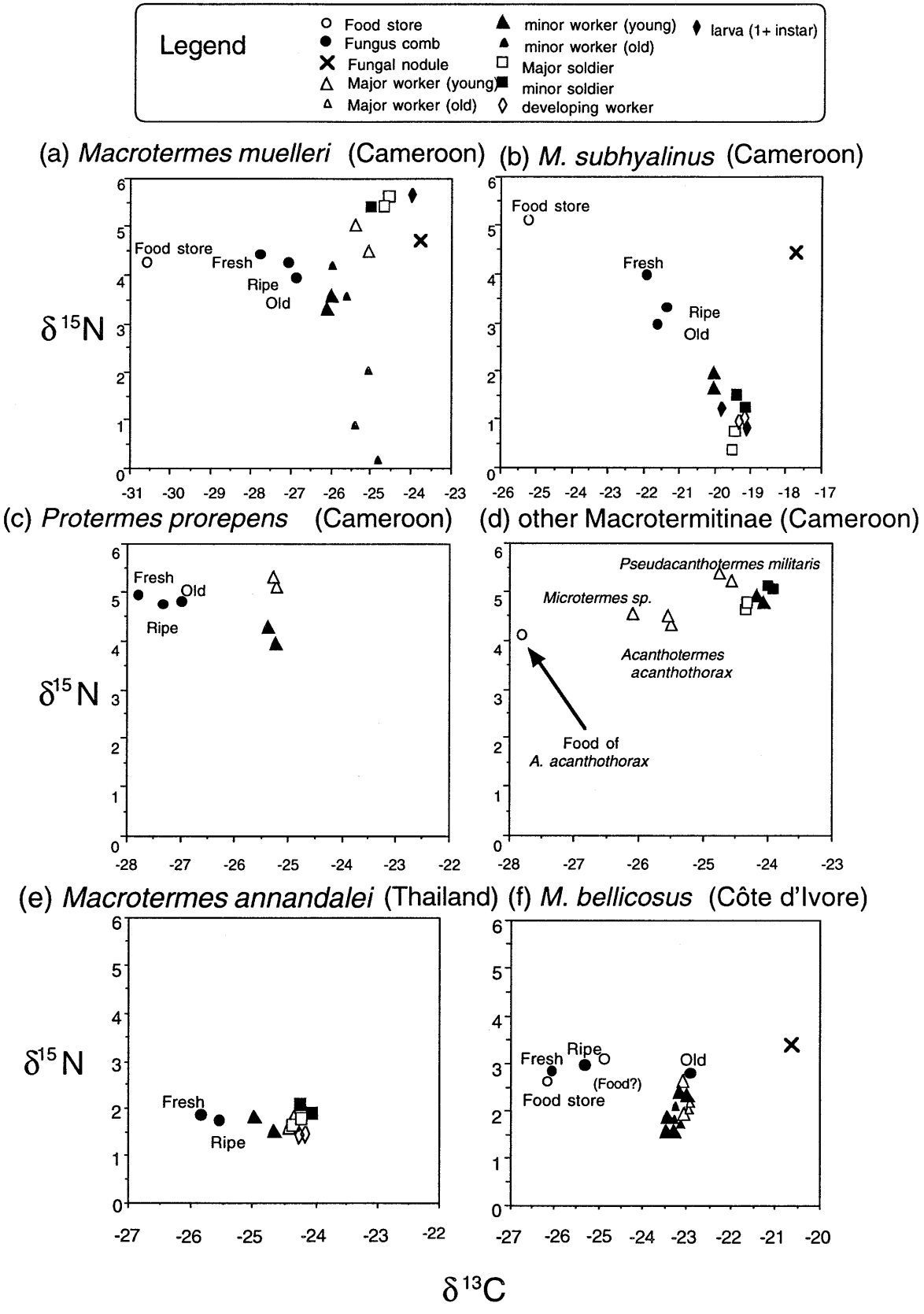


Fig. 21 $\delta^{13}C$–$\delta^{15}N$ map of fungus growing termites in Cameroon (a)-(d), Thailand (e) and Côte d'Ivore (f). Food, food store, fungal nodule and fungal comb are shown in the average value of triplicates, and termites are plotted individually.

Cameroon (a)-(d), in Thailand (e) and in Côte d'Ivoire (f).

Gradual enrichment in carbon isotope ratios from food store to termites and to fungal nodules were observed in all termite species (Boutton 1983, Tyler et al. 1988). Whereas, $\delta^{15}N$ values of termites were similar to those of food and fungal comb except for *M. subhyalinus* (**Fig. 21(b)**). A wide range of ^{15}N variation was observed only in old minor worker of *M. muelleri* (**Fig. 21(a)**). Strong trend of ^{15}N depletion was observed in every caste of *M. subhyalinus* (**Fig. 21(b)**).

6.1.3 Discussion

At this stage, the isotopic distribution of $\delta^{13}C$ and $\delta^{15}N$ in fungus growing termites cannot be explained. However, some implications and speculations could be made corresponding with some existing papers.

Differences in $\delta^{15}N$ among castes

The large differences in $\delta^{15}N$ of old minor worker of *M. muelleri* may be explained by the accumulation of uric acid (Chappell and Slaytor 1993). The major nitrogenous waste product in many phytophagous insect is uric acid. Uric acid is the end product of nucleic acid metabolism and not recycled by the insects to any extent (Lafont and Pennetier 1975). Termites accumulate fatty acid in the fat body, which is observed by the changes in color as the aging of workers. Since uric acid is stored in the fat body, the accumulation of the fat body might explain the isotopic difference among castes because the $\delta^{15}N$ of synthesized uric acid is expected to be low. While uric acid degrading bacteria exists in the hindgut, no uricase has been found in termites (Potrikus and Breznak 1980c, 1981). On the other hand, necrophagy and cannibalism are often seen in termites, which confirm the possibility that the accumulation of the uric acid in the termite body serves as the nutrient storage of nitrogen (Slaytor and Chappell 1994). Anyway, the difference in $\delta^{15}N$ might be caused by the caste selective transfer of nitrogen within a colony.

In the case of *M. subhyalinus*, however, this caste selective transfer cannot explain the pattern. One of possible causes of the low $\delta^{15}N$ is nitrogen fixation.

Nitrogen fixation

In fungus growing termites, the method utilized in Chapter 3 cannot be applied directly, because the food (fungus comb) itself is a fecal pellet of foraging workers whereby its stoichiometric mass balance is not clear. However, if this first step does not cause large fractionation (i.e. the duty of foraging workers is collecting food sources and making fungal comb), this method could be applied to fungus growers. $\delta^{15}N$ of termites of *M. annandalei* (Thailand), *M. bellicosus* (Côte d'Ivoire), *M. muelleri* and *P. prorepens* (Cameroon) were

similar to that of fungal comb, showing that nitrogen fixation was not operating in these species. However, in the case of *M. subhyalinus*, $\delta^{15}N$ of termites was lower than that of fungus comb, and the result suggests nitrogen fixation although nitrogen fixation has not been reported in the Macrotermitinae except for Nakamura and Yara (in prep.) (**Table 1**).

$\delta^{13}C$ among components

For all cases, $\delta^{13}C$ varied with the trend: stored food< New comb< Ripe comb<Old comb< Termites< Fungal nodule, which implies the selective decomposition of material.

The difference between $\delta^{13}C$ of fungal nodules and stored food was 6-8‰. This may be consistent with Gleixner et al. (1993), who demonstrated that fungi (Basidiomycetes) had about 4‰ higher $\delta^{13}C$ than that of wood tissue that they decomposed. The chitin of the fungi was enriched by 1.5 to 2 ‰ relative to cellulose, independent of whether cellulose (soft rot) or cellulose and lignin (white rot) were substrates. They hypothesized that it was due to "light" $\delta^{13}C$ of respiratory CO_2.

6.2 Wood-feeders in Sydney and grass-harvesters in Townsville

I applied ^{15}N natural abundance method to the field study at Sydney and Townsville in Australia.

6.2.1 Sampling sites

Sampling site in Sydney was warm temperate forest in the Forestry Commission of N.S.W., which is located in about 30km to the north-west of the central Sydney (**Fig. 22**). Wood-feeding termites of *Porotermes adamsoni* (Colony I and II) (Termopsidae) and *Kalotermes pallidinotum* (Colony II) (Kalotermitidae) were collected together with nesting wood, both of which were 40 cm in diameter (**Table 13**). Both species were found together in Colony II.

Sampling site in Townsville was semi-arid tropical woodland consisted of *Eucalyptus* in the field of C.S.I.R.O. about 40km to the south-west of Townsville (**Fig. 22**). Mound building termites of *Tumulitermes pastinator* (Colony III), *Drepanotermes perniger* (Colony IV), *D. rubriceps* (Colony V)

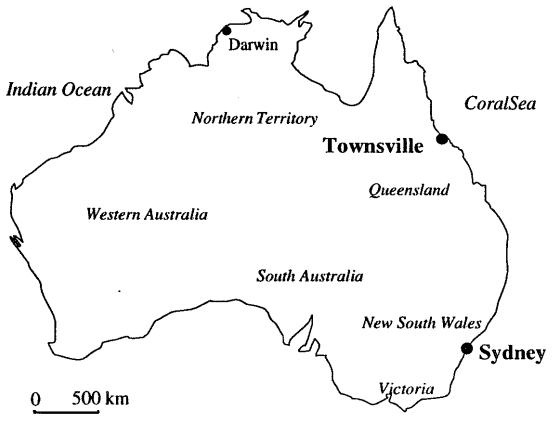


Fig. 22 Study sites in Australia
Townsville — 19°30' S, 147° E
Sydney — 34° S, 151° E

and *Nasutitermes magnus* (Colony VI) (Termitidae, Isoptera) were collected together with their stored foods, which consisted of grass that were cut into pieces (**Table 13**). *P. adamsoni* and *K. pallidinotum* have one worker caste and one soldier caste ,whereas *T. pastinator, D. perniger, D. rubriceps* and *N. magnus* have two (major and minor) worker castes and one soldier caste.

6.2.2 Results

Results of the analyses are shown in **Table 13**. Except for colony VI ($\delta^{15}N$ = 5.0), $\delta^{15}N$ of diet (wood or grass) was close to zero ($\delta^{15}N$ = -1.8 to 1.0‰).

Relationships between $\delta^{15}N$ of diet and termites are shown in **Fig. 23**. $\delta^{15}N$ of some species was higher than that of their diet, and others were not.

6.2.3 Discussion

$\delta^{15}N$ of termites collected in Sydney and Townsville

As shown in **Fig. 1**, $\delta^{15}N$ generally increases by 3.4‰ with each trophic level due to the isotope effect, while *N. koshunensis* showed another pattern. Termites in this study showed different pattern.

Diet of *N. magnus* (Colony VI) showed high $\delta^{15}N$, which must reflect the high $\delta^{15}N$ of soil nitrogen because it was collected in cattle-grazing property. Since the difference of $\delta^{15}N$ between diet (5.0±0.4‰) and atmospheric nitrogen (from 0 to -2‰) was large, it is

Table 13 $\delta^{15}N$ values of termites in Australia.
Samples were collected at warm temperate forest in Sydney (I, II) and at woodland in Townsville (III-VI). $\delta^{15}N$ of nesting wood tissue are shown in I and II, and $\delta^{15}N$ of stored grass in III-VI as a food, which are shown in the averaged values (mean±s.e.(‰)) of duplicates.$\delta^{15}N$ of workers (including pseudergates) and soldiers are shown in the averaged values (mean±s.e.(‰)), where measurement numbers are shown in the parenthesis. The upper and lower figures of $\delta^{15}N$ of workers show that of major and minor workers respectively. In each measurement, several workers or soldiers were put together.

	Species	Sampling site	$\delta^{15}N$ of wood tissue(W), grass(G)(‰)	$\delta^{15}N$ of workers (‰)	$\delta^{15}N$ of soldiers (‰)
I	*Porotermes adamsoni*	Sydney	1.0±0.4(W)	-0.3±0.3 (3)	-0.6±0.0 (2)
II	*Porotermes adamsoni*	Sydney	-1.8±0.1 (W)	1.7±0.1 (2)	0.2±0.2 (2)
	Kalotermes pallidinotum			-1.5±0.0 (2)	-1.8 (1)
III	*Tumulitermes pastinator*	Townsville	0.3±0.3 (G)	-0.1±0.0 (2)	-0.6 (1)
				0.9±0.0 (2)	
IV	*Drepanotermes perniger*	Townsville	0.8±0.1 (G)	1.3±0.0 (2)	0.9±0.1 (2)
				0.7±0.1 (2)	
V	*Drepanotermes rubriceps*	Townsville	0.0±0.2 (G)	-0.8+0.0 (2)	0.7±0.1 (2)
				0.4 (1)	
VI	*Nasutitermes magnus*	Townsville	5.0±0.4 (G)	7.4±0.1 (2)	6.8±0.0 (2)
				7.0±0.0 (2)	

obvious that this species scarcely used atmospheric nitrogen by using the equation (6). Assuming that $0‰<\Delta_{dig}<5‰$ and $-2‰<\Delta_{fix}<0‰$, 0–30% of $\%N_{dfa}$ was obtained (**Table 14**), where I assumed that the upper limit of the isotope effect during digesting wood tissue is 5‰ referring to the maximum range of isotope effect in **Fig. 1**. Although these assumptions of both fractionation factor ranges widely, there is no indication that they use atmospheric nitrogen as much as that observed in *N. koshunensis*.

As for other termites that fed on grasses in Townsville (Colony III, IV, V), it was more difficult to conclude what proportion of nitrogen came from the atmosphere. It is because the $\delta^{15}N$ of diet was close to zero and because difference among castes, especially major and minor worker, was large (**Table 14**). For example, using $\delta^{15}N$ of major workers of *T.*

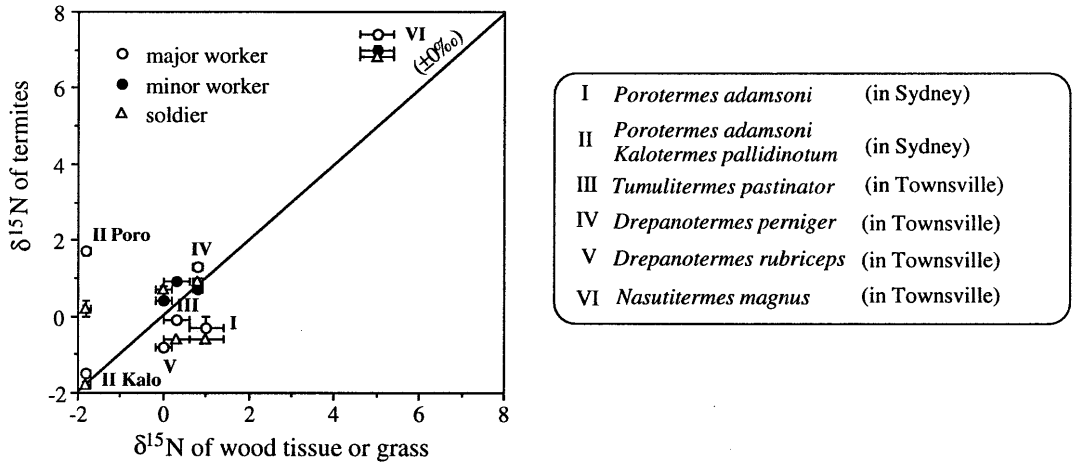


Fig. 23 Relationship between $\delta^{15}N$ of diet (‰) and $\delta^{15}N$ of termite (‰) collected in Sydney (solony I and II) and Townsville (colony III to VI). I and II are wood feeders in the forest, while III to VI are grass feeders in the woodland. Open circle, closed circle and open triangle denote major worker, minor worker and soldier, respectively, shown with standard error. When no isotopic shift occurs during digestion, points would be on the line of slope 1 shown as (±0‰).

Table 14 The fraction of nitrogen derived from atmosphere(%Ndfa) calculated from the data of (major) workers (Table 13) with the ranges of Δ_{dig} and Δ_{fix} in the text; $0‰<\Delta_{dig}<5‰$, $-2‰<\Delta_{fix}<0‰$.

Colony	Species	Ranges of %Ndfa (%)
I	*Porotermes adamsoni*	80-100
II	*Porotermes adamsoni*	0-50
	Kalotermes pallidinotum	#
III	*Tumulitermes pastinator*	20-100
IV	*Drepanotermes perniger*	#
V	*Drepanotermes rubriceps*	40-100
VI	*Nasutitermes magnus*	0-30

The range of %Ndfa cannot be estimated with the assumption of the ranges of Δ_{dig} and Δ_{fix}.

pastinator (Colony III) and *D. rubriceps* (V), $\%N_{dfa}$ ranges 20–100% and 40–100% respectively. Whereas, using $\delta^{15}N$ of minor workers, $\%N_{dfa}$ cannot be estimated with the assumed ranges of $0‰<\Delta_{dig}<5‰$ and $-2‰<\Delta_{fix}<0‰$. $\%N_{dfa}$ cannot be estimated in *D. perniger* (Colony IV), using $\delta^{15}N$ of major nor minor worker.

P. adamsoni (Colony I) had lower $\delta^{15}N$ values than that of diet, suggesting that they used much atmospheric nitrogen. *P. adamsoni* and *K. pallidinotum* had different isotope ratios although they nested in the same wood (Colony II). This difference might be due to the different nitrogen metabolism of the two species, or only due to the heterogeneity of their diet within the nesting wood. The latter explanation is plausible, since the nesting wood of *P. adamsoni* and *K. pallidinotum* , which was about 40cm in diameter, lay on the forest floor and was partly decomposed, whereas those of *N. koshunensis*, which were less than 7cm in diameter, were dead branches of living trees and scarcely decomposed.

7. GENERAL DISCUSSION

7.1 Nitrogen fixation in various termites – its pattern and implication by applying $\delta^{15}N$ natural abundance method

Integration of $\delta^{15}N$ data for the estimation of nitrogen fixation in termites

I compared the contribution of nitrogen fixation in various termite species that have been studied in the thesis, except for fungus growing termites in the section 6.1. I compared $\delta^{15}N$ of worker (in the case there were two morphotypes in the worker caste, I selected the major one only for convenience) against $\delta^{15}N$ of diet or other material that had a representative $\delta^{15}N$ value of diet (**Fig. 24**). $\delta^{15}N$ of workers and diet that I assumed are listed in Appendix: **Table A1**. Basically, wood materials were identified as diet in wood feeding and wood/soil interface feeding species, while mound materials in soil feeding species, where each categorization are based on Chapter 3 to 6. In some species, however, other materials were substituted for, in the case when identified diet had not been available (**Table A1**). In **Fig. 24**, diagonal line denotes $\Delta\delta^{15}N=0$, i.e. the line when no isotope effect occurs, and shaded area shows the region of $\delta^{15}N$ acquired through nitrogen fixation. The region between the diagonal line and the shaded region shows the area where nitrogen fixation is important to a certain extent in the nitrogen economy of the species.

The result showed that nitrogen fixation was very important in *Neotermes koshunensis* and *Cryptotermes secundus*, while important to a certain extent in *Microcerotermes parvus*, on the other hand, not important in other termite species especially soil-feeding termites (**Fig. 24**).

Fig. 24 also exhibited three levels of isotope effects, which accounted for wood-feeders, wood/soil interface feeders and soil-feeders, respectively. There is uncertainty in $\delta^{15}N_{diet}$–$\delta^{15}N_{termite}$ plot caused by the uncertainty in identifying the diet, especially in soil feeders. However, it is easier to compare various termite species irrespective of sites, excluding the variation of background $\delta^{15}N$ of soils.

Nitrogen metabolism in termites

Ii is required to compare the contribution of nitrogen fixation in relation to degree of decomposition, carbon and nitrogen content of the diet, and life types (one piece type or separates type).

Since it costs much energy to reduce atmospheric nitrogen (N≡N) to ammonia (NH_3), it can be reasonable that nitrogen fixation occurs inversely proportional to nitrogen content of

the diet. In fact, the high concentration of ammonia in the paunch of *Nasutitermes walkeri* (3mM), calculated using the paunch volume, suggests that fluxes in NH_4^+ concentration may control the activity of nitrogenase in the symbiotic bacteria (Slaytor and Chappell 1994).

Nitrogen fixation was significantly important to wood-feeders especially lower termites, whereas scarcely contributed to nitrogen economy in grass harvesters, wood-soil interface feeders and soil-feeders (**Fig. 24**). Nitrogen content in wood tissue and grass is shown in **Table 15**. The difference in nitrogen content of wood tissue and grass would explain the difference in $\Delta\delta^{15}N$ pattern (**Fig. 24**), suggesting the difference in the contribution of N_2 fixation. Although the contribution of nitrogen fixation is not inversely proportional to nitrogen content of the diet directly, the tendency of inverse relationship

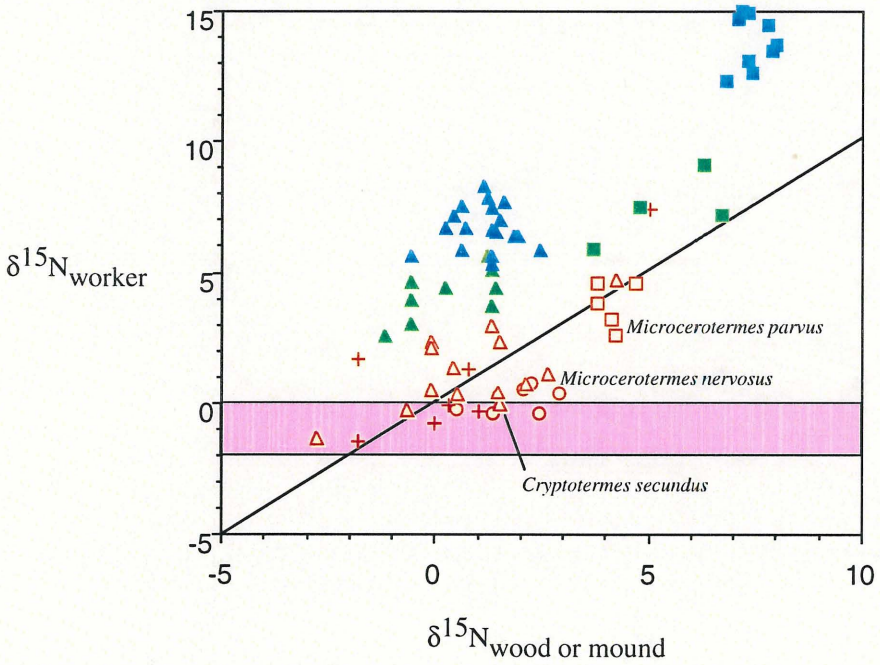


Fig. 24 Comparison between $\delta^{15}N$ of worker (in the case there are two morphotypes in the worker caste, I selected the major one) against $\delta^{15}N$ of diet. I selected wood tissue for food in wood-feeders and wood/soil interface feeders, but mound material for soil-feeders which were identified in Chapter 3 to 6. $\delta^{15}N$ of each colony and selected food material were listed in Appendix: Table A1. Diagonal line denotes $\Delta\delta^{15}N=0$, i.e. the line when no isotope effect occurs, and shaded area shows the region of $\delta^{15}N$ acquired through nitrogen fixation.

Table 15 Nitrogen content of wood and grass for the food of termites (Ash weight was included in total weight).

Species	Colony number	Nitrogen Content weight %	
		Wood(W) or Grass(G)	Feces
Neotermes	1	0.31 (W)	—
koshunensis	2	0.13 (W)	—
	3	0.12 (W)	0.27
	4	0.11 (W)	0.43
	5	0.12 (W)	0.28
	6	0.070 (W)	0.35
	Experiment	0.11 (W)	—
I	*Porotermes adamsoni*	0.054 (W)	—
II	*Porotermes adamsoni*		
	Kalotermes pallidinotum	0.12 (W)	—
III	*Tumulitermes pastinator*	0.48 (G)	—
IV	*Drepanotermes perniger*	0.36 (G)	—
V	*Drepanotermes rubriceps*	0.97 (G)	—
VI	*Nasutitermes magnus*	0.35 (G)	—

between them was confirmed, which had been reported by Prestwich et al. (1980). On the other hand, as discussed in Chapter 4 and 5, carbon content in soil organic matter (**Table 8, 11**) must limit nitrogen fixation in the gut of soil-feeding termites since nitrogen fixation requires much carbon as energy source. The fact that low nitrogen and enough carbon lead to the use of nitrogen fixation in termites partly supports C/N balance hypothesis (Higashi et al. 1992), although the carbon and nitrogen sources of soil-feeders are still unclear.

From the evolutional point of view, termites are supposed to evolve from lower termites, of which intestinal microbiota includes protozoa as well as bacteria, to higher termites, of which the microbiota consists of bacteria alone. Yamaoka (1989) concluded that symbiotic protozoa (flagellates) played an indispensable role in cellulose digestion of lower termites. Inoue et al. (1997) showed a lower termite, *Reticulitermes speratus*, had the ability to degrade cellulose to a certain extent. On the other hand, Veivers et al. (1991) has reported that at least 99% of cellulase was termite origin in Macrotermitinae (higher termites). Most of lower termites, except for the Hodotermitidae, are wood-feeders, while higher termites includes various kinds of feeding habits: wood-feeders, grass-feeders, fungus growers, wood/soil interface feeders and soil-feeders. It seems that wood-feeding habit, together with nitrogen fixation, is an efficient strategy in utilizing limited resources especially in one-piece type termites, which was observed in *Neotermes koshunensis* and *Cryptotermes secundus*. But, nitrogen fixation requires much energy. Therefore, once termites, especially higher termites, came to use grass or fungi that was rich in nitrogen, there would be no need to use atmospheric nitrogen. The finding that only some wood-feeding termites, especially lower termites, were dependent on nitrogen fixation corresponds to the trend that termites evolved

to be less dependent on gut microbes. The evolution from lower to higher termites may have followed the prosperity of grassland in the Late Cenozoic. Nitrogen fixation didn't occur in *Macrotermes ukuzii* (Rohrmann and Rossman 1980) and scarcely contributed to *Odontotermes formosanus* (Nakamura and Yara in prep.) and *Hodotermes mosambicus* (Hewitt et al. 1987), confirming the pattern mentioned above.

Sylvester-Bradley et al. (1978), Prestwich et al. (1980), Prestwich and Bentley (1981), Bandeira (1983) and Lovelock et al. (1985) have reported the high rate of nitrogen fixation in *Nasutitermes* spp.. However, the significance of nitrogen fixation in the wood-feeding Nasutitermitinae could not be estimated because there were not enough data sets, and requires to be studied.

7.2 Stable isotope ratios in detritivorous animals

Nitrogen isotope effect as an indicator of feeding habit on humification gradient

I observed wide range of nitrogen isotope effect ($\Delta\delta^{15}N$) from -1.6 to 8.8 ‰ which reflects the diversity of nutrient acquisition from plant debris in the wood to humus in the soil (Chapter 4 and 5). There are some papers that reported wide isotope effect in detritivorous animals. For example, Kikuchi and Wada (1996) reported 5±1‰ for a deposit feeder, *Neanthes japonica* (Polychaeta, Annelida). Fry (1988) also observed about $\Delta\delta^{15}N$=6‰ for Polychaete, Isopods and Squid. Whereas there are no references that studied isotope effects along with humification gradient in one animal group. In other words, the present study is the first report that studied isotope ratios among one group where less physiological differences exists in the phylogenetical sense. Although there are not enough data to discuss about general "humus or deposit" feeders, this method may be applicable to general detritivorous animals as well as termites in identifying the diet .

Minagawa and Wada (1984) pointed out the simple relationship that $\delta^{15}N$ was enriched around 3.4‰ per each trophic level. The idea of isotope effect is basically based on the grazing food chain. However, in this study, I showed that the isotope effect was different at the bottom of the detritus food chain. I have shown the isotope effect only in termites, whereby, general survey in detritivorous animal is required in the sentence of isotope ecology.

Intestinal interactions among symbionts and feeding habit

I have shown that $\delta^{15}N$ ($\Delta\delta^{15}N$) is a good indicator to distinguish soil-feeders from wood-feeders, but the evolution of soil-feeding must be considered in relation to cellulose digestibility. Inoue et al. (in prep.) reported the significant cellulase activity in the hindgut of *Macrognathotermes sunteri*, which I concluded one of the typical soil feeders at Darwin

region. They suggested that amoebae in the hindgut might contribute to the cellulase activity. On the other hand, Rouland et al. (1986) reported no cellulase activity in African soil-feeding termite, *Thoracotermes macrothorax*. It is required to compare the nitrogen economy and cellulose digestibility in African region as well as in Australian region. $\delta^{13}C$ between wood-feeders and soil-feeders were also distinguishable, partly which may have been caused by the carbon source.

Some studies have suggested that intestinal interaction may influence isotope effect. Sealy et al. (1987) suggested that $\delta^{15}N$ might be enriched if nitrogen was recycled among microbes in rumen. Sutoh et al. (1987) reported 2.8-5.6‰ enrichment of $\delta^{15}N$ between protozoa and bacteria, where the former were assumed to digest the latter, in the rumen of cattle.

7.3 Termite–symbionts system by stable isotope techniques

Nutritional transfer among castes observed by $\delta^{15}N$

The $\delta^{15}N$ of soldiers was lower than that of workers in wood and grass feeders **(Fig. 6 and 23)**. There are two possible explanations. First, soldiers could fix nitrogen more than workers and/or it could be attributed to the caste-selective nitrogen transfer from gut microbes to termite that Bentley (1984) reported. Since nitrogen fixation rate among castes were different from species to species (Prestwich et al. 1980), difference between castes may reflect the fixing pattern. Second, workers could give soldiers some selected ingredients, of which $\delta^{15}N$ is lower than that of wood tissue.

However, $\delta^{15}N$ of soldiers in soil-feeding termites were both lower and higher than that of workers **(Fig. 13)**. Several different pattern were also found from species to species in fungus growers **(Fig. 21)**. Caste selective patterns of $\delta^{15}N$, combined together with $\delta^{13}C$ (*cp*; Sugimoto et al. in prep.), has the information of intraspecific as well as interspecific food habits, and needs to be studied with reference to sociality and nutrition.

I measured $\delta^{15}N$ of whole body of termites, and then that of separated guts with symbionts and termite body **(Fig. 16)**. If it is possible to measure gut symbionts separately, nutritional interaction between termites and symbionts may be more understood (Bentley 1984).

Termites in nutrient cycling

Termites, which are predominant soil animals in the tropical terrestrial ecosystems, consume 24-32% of annual supply of fallen leaves in Malayan tropical forest (Matsumoto and Abe 1979), and 63% of grass litter in Nigerian savanna (Wood and Sands 1978), and are

preyed on by various animals. Since these are mainly separate-type termites that harvest grass or leaf litter, which were proved to be less nitrogen fixer (Section 6.2), an interesting possibility that nitrogen fixation of termites plays an important role in the terrestrial ecosystem may have been abandoned. The termites which utilize atmospheric nitrogen as a significant nitrogen source are mainly one-piece type wood-feeders, whereby nitrogen fixation in termites is comparatively less important to the global nitrogen cycling. On the other hand, *Neotermes* and *Cryptotermes*, in which I confirmed high nitrogen fixing ability, are abundant especially in mangrove forest. There is still a possibility that termites play a significant role in the food web of such ecosystems.

Termites, which are comparable to earthworms (Oligochaeta) and springtails (Collembola) in temperate region, are abundant in tropical region (Swift et al. 1979). The prosperity of termites are mainly due to higher termites (Noirot 1992) especially fungus growers in savanna and soil feeders in woodland and rain forest. The information of production of soil-feeders is much less than that of foraging termites, such as grass harvesters including fungus growers (for example, Ohiagu 1979a, b). Ecological observation is still required to soil-feeders.

Termite-Symbionts system from stable isotope point of view

The termite-symbionts system, which is one of the exquisite endo- and exo-symbionts system, can be studied by stable isotope techniques. Since the intestinal interactions include carbon and nitrogen transfer among microbiota as well as between termite and microbes, the isotopic effect gives us ecological together with physiological information of termite-symbionts system. Finally, I have shown two schematic figures that are useful to the study of Termite-Symbionts system using the stable isotope techniques in Appendix section. **Fig. A1** shows the intestinal interaction between termite and symbionts with stable isotope ratios and isotope fractionation, and **Fig. A2** shows the illustrative position of termites in the $\delta^{13}C$–$\delta^{15}N$ plain.

8. CONCLUSION

The natural abundance method is appropriate for the estimation of N_2 fixation under natural condition, because it is free from manipulation, although it is not so sensitive unless the fractionation factor is measured. The most significant point in applying the ^{15}N natural abundance method to the study of nitrogen fixation in termites consists in its initial dependency on $\delta^{15}N$ of the diet. Diet must be identified, $\delta^{15}N_{diet}$ should be distinguishable from $\delta^{15}N$ via nitrogen fixation ($\delta^{15}N$=0‰ to -2‰) and positive $\delta^{15}N_{diet}$ is favorable to estimate the contribution of N_2 fixation, which requires less assumption of the fractionation factors.

Nitrogen isotope effect, $\Delta\delta^{15}N$, is an indicator of functional position of the species in the humification process. $\Delta\delta^{15}N$ of wood-feeders are nearly zero, whereas those of soil-feeders are five to nine, wood/soil interface feeder being in intermediate. $\delta^{15}N$ of worker caste is available to distinguish the feeding habit in the humification process unless background of $\delta^{15}N$ values varies significantly.

$\delta^{13}C$ also distinguishes wood-feeders from soil-feeders. The reason lies possibly in the different intestinal interaction between termites and microbes as well as in the difference of food materials in the pedogenic process.

Nitrogen fixation plays an important role in wood-feeding termites, especially one-piece type lower termites, whereas scarcely contributes to nitrogen economy in grass harvesters, wood/soil interface feeders and soil-feeders. This is probably due to the high C/N ratio of wood, which provides enough energy to fix atmospheric nitrogen, and which contains low nitrogen not to suppress nitrogen fixation.

This is the first report that studied the termite–symbionts system using the nitrogen stable isotope techniques, which enabled to put termite species in position numerically on the the humification gradient. This technique has revealed the feeding habit of termites and suggests a possibility to be applicable to detritivorous animals in general.

Molecular phylogenetical study together with stable isotope study confirmed the diversification in feeding habit of termite species, especially *Termes-Capritermes* species of the Termitinae in Australia.

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SYNOPSIS (IN JAPANESE)

——摘要——

京都大学大学院理学研究科生物科学専攻動物学系博士申請論文
「安定同位体を用いた、シロアリー微生物共生系の栄養生態学的研究」
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食材性シロアリが、わずかの窒素しか含まない餌を利用して生きていける理由を調べるために、シロアリー微生物共生系の窒素固定能を研究した。沖縄県西表島において採取されたコウシュンシロアリの、餌材とシロアリ個体の窒素安定同位体比（$\delta^{15}N$）を比較したところ、個体の$\delta^{15}N$は材の$\delta^{15}N$より低い値を示した。これにマメ科根粒菌の窒素固定能の推定に用いられてきた方法を応用すると、体の窒素の少なくとも30%から60%が窒素固定由来であることがわかった。この結果は、トレーサー実験によって確認された。

アフリカ中央部のカメルーン、ムバルマヨ保護林において採集されたシロアリ群集の安定同位体比を分析した。その結果、シロアリの$\delta^{15}N$は大きな変異を示していた。$\delta^{15}N$は、食材性からよく腐朽した材食（中間食性）を経て土壌食性に至る勾配に対応して上昇しており、食性が$\delta^{15}N$を用いて研究できることがわかった。また、餌とシロアリの$\delta^{15}N$の差である同位体効果の値を用いた場合、$\Delta\delta^{15}N$= -1.6 から +8.8‰、$\Delta\delta^{13}C$= -2.2 から +3.0‰という広い値になっており、シロアリと体内共生微生物の多様な関係を示唆していた。

また、オーストラリア北部のダーウィンで採集されたシロアリ亜科について上記方法の食性調査と系統関係の分析を行った。その結果、腐植食性は*Termes-Capritermes*族群で一回進化したと考えられた。更に、中間食性ー土壌食性に関わる種群がカメルーン・オーストラリア両地域で異なり、それぞれの地域の腐植食性の進化の特徴を示していた。

続いて、この方法をキノコシロアリについて適用したところ、カースト別に異なる同位体比を示し、カースト別の物質のやり取りを反映していることが示唆された。

最後に、この方法を用いて種々のシロアリにおいての窒素固定の寄与について調べてみたところ、一般に土壌食性や草食性では窒素固定からの寄与がほとんどなかった。結論として、食材性のシロアリで特異的に高いほかは、シロアリー微生物共生系における窒素固定能は低いことが示された。

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APPENDIX

Table A1* $\delta^{15}N$ of termites and associated substrates mentioned in the thesis (See each chapter for details).

Sampling site / Species	Colony number#	$\delta^{15}N_{wood/grass}$	$\delta^{15}N_{soil}$	$\delta^{15}N_{nest/mound}$	$\delta^{15}N_{worker}$	
Iriomote (Chapter 3)						
Neotermes koshunensis	1	**1.3**§			**-0.4**§	W¶
	2	**2.9**			**0.3**	W
	3	**0.5**			**-0.2**	W
	4	**2.0**			**0.6**	W
	5	**2.2**			**0.8**	W
	6	**2.4**			**-0.4**	W
Sydney (Chapter 6)						
Porotermes adamsoni	I	**1.0**			**-0.3**	W
Porotermes adamsoni	II	**-1.8**			**1.7**	W
Kalotermes pallidinotum	II	**-1.8**			**-1.5**	W
Townsville (Chapter 6)						
Tumulitermes pastinator	III	**0.3**			**-0.1**	L/G
Drepanotermes perniger	IV	**0.8**			**1.3**	L/G
Drepanotermes rubriceps	V	**0.0**			**-0.8**	L/G
Nasutitermes magnus	VI	**5.0**			**7.4**	L/G
Cameroon (Chapter 4)						
Cubitermes fungifaber	1				15.4	
Cubitermes heghi	1	6.6	6.2	**7.2**	**15.0**	S
Cubitermes heghi	2	4.8	7.1	**7.3**	**14.9**	S
Thoracotermes macrothorax				**7.1**	**14.7**	S
Cubitermes fungifaber	2	6.0	7.4	**7.8**	**14.5**	S
Cubitermes fungifaber	3			**8.0**	**13.7**	S
Procubitermes arboricola			6.2	**7.9**	**13.5**	S
Astalotermes sp. nov.		3.0	**7.3**		**13.1**	S
Jugositermes tuberculatus		4.2	**7.4**		**12.6**	S
Astalotermes quietus		5.1	5.9	**6.8**	**12.3**	S
Termes hospes	1	**6.3**			**9.1**	W/S
Termes hospes	2	**4.8**		4.9	**7.5**	W/S
Nasutitermes latifrons		**6.7**		6.2	**7.2**	W/S
Cephalotermes rectangularis		**3.7**		3.5	**5.9**	W/S
Microcerotermes parvus	1	**4.7**			**4.6**	W
Microcerotermes parvus	2	**3.8**			**4.6**	W
Microcerotermes parvus	3	**3.8**			**3.8**	W
Microcerotermes parvus	4	**4.1**			**3.2**	W
Acanthotermes acanthothorax					4.4	
Microcerotermes parvus	5	**4.2**			**2.6**	W

Continue to the next page

*$\delta^{15}N$ values are averaged value of more than tripricates.

#Colony numbers in Cameroon and Darwin were numbered in $\delta^{15}N_{worker}$ order.

§Numbers with bold characters were shown in $\delta^{15}N_{wood\ or\ mound} - \delta^{15}N_{worker}$ plot (Fig. 24, Section 7.1).

¶Feeding habits used in Fig. 24, which were identified in the chapters 3 to 6. W: wood feeder, L/G: litter or grass harvester, W/S: wood/soil interface feeder, S: soil feeder.

Table A1 (Continued)

Sampling site / Species	Colony number	$\delta^{15}N_{wood/grass}$	$\delta^{15}N_{soil}$	$\delta^{15}N_{nest/mound}$	$\delta^{15}N_{worker}$	
Darwin (Chapter 5)						
Mastotermes darwinisnsis	1				0.4	
Mastotermes darwiniensis	2				2.0	
Cryptotermes secundus	1	**1.6**			**-0.2**	W
Cryptotermes secundus	2	**1.6**			**-0.1**	W
Cryptotermes secundus	3	**0.6**			**0.2**	W
Cryptotermes secundus	4	**1.4**			**0.4**	W
Cryptotermes secundus	5	**2.2**			**0.6**	W
Coptotermes acinaciformis					2.3	
Heterotermes vagus		**0.0**			**2.0**	W
Schedirhinotermes actuosus				**1.6**	**2.2**	W
Schedorhinotermes breinli					1.6	
Microcerotermes boreus				**0.5**	**1.2**	W
Microcerotermes nervosus	1			**-0.6**	**-0.4**	W
Microcerotermes nervosus	2			**0.0**	**0.4**	W
Microcerotermes nervosus	3			**2.7**	**1.0**	W
Amitermes arboreus	1			**-0.5**	**2.9**	W/S
Amitermes arboreus	2	**-0.5**	0.4		**4.5**	W/S
Amitermes laurensis		**0.0**		2.0	**2.2**	L/G
Amitermes perelegans	1		**1.4**		**5.2**	S
Amitermes perelegans	2			**2.5**	**5.7**	S
Amitermes subtilis				**4.3**	**4.6**	L/G
Xylochomitermes melvillensis	1	**-1.1**	0.8		**2.4**	W/S
Xylochomitermes melvillensis	2	**1.4**		-0.1	**3.6**	W/S
Xylochomitermes melvillensis	3	**1.5**			**4.3**	W/S
Xylochomitermes melvillensis	4	**1.4**			**5.0**	W/S
Ephelotermes melachoma	1	**-0.5**		0.0	**3.8**	W/S
Ephelotermes melachoma	2			**0.3**	**4.3**	W/S
Ephelotermes melachoma	3	**1.3**		1.6	**5.5**	W/S
Cristatitermes carinatus			**1.4**		**5.5**	S
Cristatitermes froggatti	1			**2.0**	**6.3**	S
Cristatitermes froggatti	2		3.1	**1.5**	**6.4**	S
Cristatitermes froggatti	3			**1.6**	**6.9**	S
Hapsidotermes orbus				**0.7**	**7.4**	S
Lophotermes quadratus				**0.5**	**7.0**	S
Lophotermes septentrionalis				**0.7**	**5.7**	S
Macrognathotermes sunteri	1			**-0.5**	**5.5**	S
Macrognathotermes sunteri	2	0.7		**1.9**	**6.3**	S
Macrognathotermes sunteri	3			**1.4**	**6.5**	S
Macrognathotermes sunteri	4			**0.3**	**6.6**	S
Macrognathotermes sunteri	5			**0.8**	**6.6**	S
Macrognathotermes sunteri	6				7.1	
Macrognathotermes sunteri	7			**1.4**	**7.3**	S
Macrognathotermes sunteri	8			**1.7**	**7.6**	S
Macrognathotermes sunteri	9			**1.3**	**7.7**	S
Macrognathotermes sunteri	10			**1.2**	**8.2**	S
Nasutitermes triodiae		**-2.7**		2.1	**-1.5**	L/G
Tumulitermes comatus				**1.4**	**2.8**	W

See Caption in the previous page

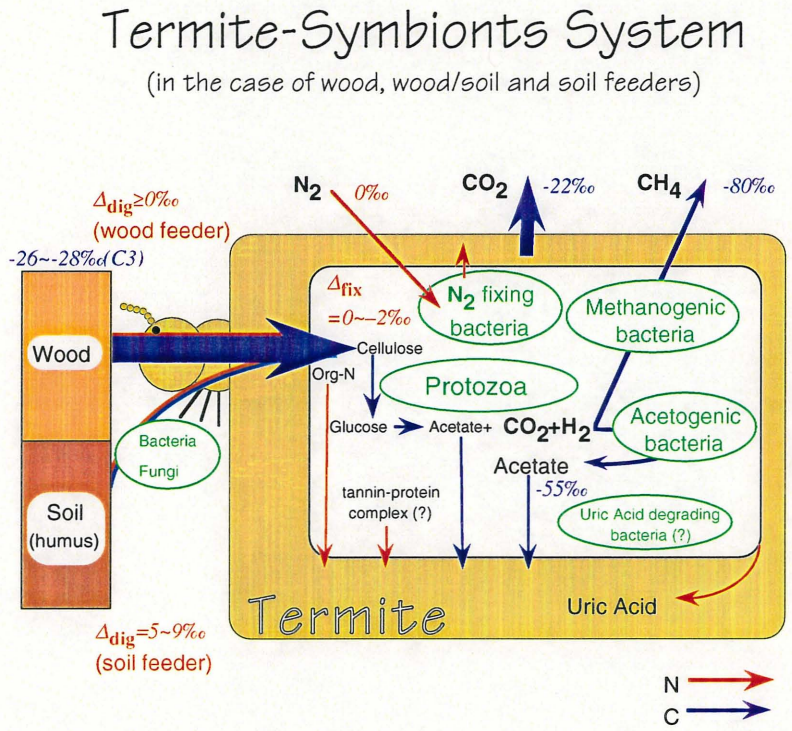

Fig. A1 The Termite-Symbionts System. Stable isotope ratios and isotope fractionation concerning with the Termite–Symbionts system are shown in the figure. Mechanisms are based on Bignell (1994), Brauman et al. (1992) and Breznak and Brune (1994). The isotope ratios are based on Tayasu et al. (1994, 1997) and Sugimoto et al. (in prep.).

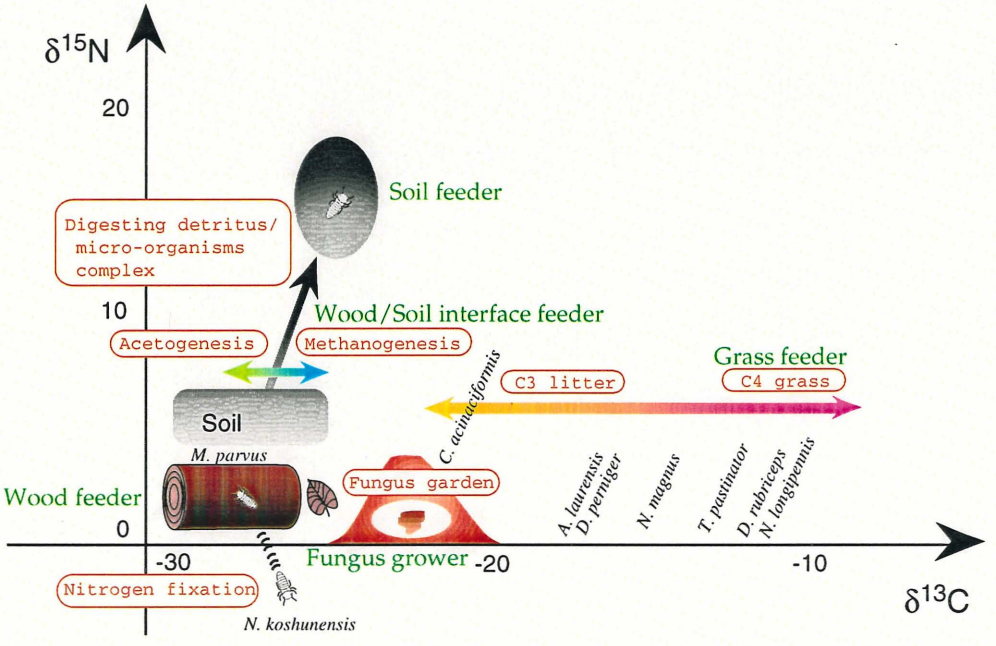

Fig. A2 Termites and associated substrates in the $\delta^{13}C$–$\delta^{15}N$ plain. Feeding habits are illustratively written in green and mechanisms that cause isotopic fractionation are written in red (based on Tayasu et al. (1994, 1997) and Sugimoto et al. (in prep.)). $\delta^{13}C$ values of termite assemblage in woodland of Townsville (QLD, Australia) were added in the figure, suggesting the feeding habit between leaf litter (C3) and grass (C4) (section 6.2).